THÈSE DE DOCTORAT D'ÉTAT

CONTRIBUTION DE L'ETUDE DE LA CINETIQUE DE LA PROLIFERATION CELLULAIRE A LA CONNAISSANCE DE LA PHYSIOPATHOLOGIE DE TROIS NEOPLASIES MURINES INDUITES PAR DES RETROVIRUS

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RESUME

NEW

Parmi les nombreuses leucémies induites par des rétrovirus chez la souris, celle provoquée par le virus de Friend polycythémiant présente des caractéristiques qui en font un modèle particulièrement intéressant pour l'étude de la cancérologie virale.

Elle est en effet rapidement induite chez la totalité des souris infectées, qui meurent environ au bout de 40 jours, en présentant essentiellement une rate hypertrophiée (poids multiplié par 30 environ), envahie de cellules leucémiques. Celles-ci, également appelées cellules hyperbasophiles, sont des précurseurs érythroides(cellules de type pro-érythroblastique). L'étude de cette néoplasie a donc bénéficié des nombreuses techniques permettant d'étudier l'érythropoièse, qui est encore actuellement le processus de différenciation hématopoiétique le mieux connu. Enfin, les cellules hyperbasophiles, contrairement à l'ensemble des cellules tumorales connues, ne peuvent généralement pas être greffées. En effet, ce n'est qu'à une phase très tardive de la maladie, et de façon exceptionnelle, que leur injection par voie sous-cutanée à des souris isogéniques donne naissance à une tumeur solide.

C'est cette dernière caractéristique qui nous a conduit à

nous interroger sur la capacité même des cellules à proliférer et sur leur durée de vie. En effet, l'augmentation progressive du nombre de cellules hyperbasophiles au cours de la maladie peut résulter soit directement de leur prolifération, soit de leur production continue à partir de cellules hématopoiétiques précurseurs. Dans cette seconde hypothèse, les cellules hyperbasophiles s'accumulent mais ne prolifèrent pas elles-mêmes, ou très peu.

Une étude approfondie de la cinétique de la prolifération des cellules hyperbasophiles nous a donc semblé nécessaire à une compréhension de l'ensemble de la maladie.

Nous avons effectué cette étude *in vivo*, au niveau de la rate. Les cellules proliférantes ont été marquées, pendant leur phase S du cycle mitotique, par la thymidine tritiée (^{3}HTdR) et nous avons déterminé sur des autoradiogrammes de sections histologiques de rate, la fraction de cellules leucémiques proliférant (exprimée par le coefficient de prolifération cellulaire, GF) et leur vitesse de prolifération (Tc). Nous avons également suivi leur devenir en les marquant par la ^{125}I-5-iodo-2'-désoxyuridine (^{125}IUdR), autre précurseur radioactif de l'ADN qui, contrairement à l'^{3}HTdR, n'est pas réutilisé après la mort des cellules.

Nous avons ainsi montré que presque toutes les cellules leucémiques prolifèrent dans la rate (Tc = 7,6 heures ; GF = 64%). Cependant, seule une très faible proportion (5%) des cellules produites

est responsable de la lente augmentation du poids de cet organe (temps de doublement : 6 jours). Près du tiers des cellules produites se différencient en érythrocytes, et environ les deux-tiers disparaissent rapidement, essentiellement par mort *in situ* après une ou deux divisions.

Ainsi, bien que proliférantes, les cellules leucémiques de Friend n'ont pas, dans leur ensemble, la capacité de s'autorenouveler ; ceci permet dont de mieux comprendre, sans toutefois l'expliquer totalement, pourquoi ces cellules ne peuvent habituellement pas être greffées.

D'autre part, ces résultats ont permis de préciser un modèle rendant compte de façon cohérente des principales données expérimentales concernant la physiopathologie de la maladie. Ce modèle s'appuie sur le fait que chez les souris présentant la leucémie de Friend, la différenciation des cellules souches précurseurs, les ERC (Erythropoietin Responsive Cells), en proérythroblastes (ou cellules hyperbasophiles) peut s'effectuer sans érythropoiétine, hormone qui dans les conditions normales permet le contrôle de cette étape de l'érythropoièse. Ce processus n'est donc plus régulé. L'érythropoièse étant un processus en équilibre instable, la différenciation permanente des ERC transformées par le Virus de Friend stimulerait leur production à partir des compartiments de cellules souches hématopoiétiques précurseurs. Nous avons montré que la presque-totalité (94%) des cellules hyperbasophiles ainsi produites disparaissent rapidement de la rate, et ne sont donc pas capables de s'autorenouveler. L'augmentation du nombre de cellules hy-

perbasophiles au cours de la maladie résulterait donc d'un excès de la vitesse de leur production par rapport à la vitesse de leur perte.

Ces caractéristiques de la prolifération des cellules leucémiques de Friend étaient très différentes de celles des autres leucémies étudiées, qui étaient principalement des leucémies greffées et non induites et dans lesquelles la perte cellulaire était presque nulle pendant la phase de croissance rapide de la tumeur.

Nous nous sommes donc demandés si les caractéristiques que nous avons décrites étaient spécifiques de la leucémie de Friend ou si elles étaient au contraire communes à l'ensemble des néoplasies murines induites par des rétrovirus.

C'est pourquoi nous avons étudié la cinétique de la prolifération cellulaire de deux autres néoplasies viro-induites :
- l'une est hématopoiétique : c'est le syndrôme myéloprolifératif induit par le MPSV (Myeloproliferative Sarcoma Virus), qui présente de nombreuses caractéristiques communes avec la leucémie de Friend (prolifération des cellules leucémiques essentiellement dans la rate, splénomégalie, évolution rapide de la maladie). Cependant, contrairement à la leucémie de Friend, les cellules néoplasiques ne sont pas exclusivement erythroïdes mais également granulocytaires, blastiques indifférenciées et de type fibroblastique.

- L'autre est une tumeur solide, c'est le rhabdomyosarcome induit par le virus sarcomatogène de Moloney (M-MSV) chez les souriceaux nouveaux-nés.

Nos résultats ont montré que, dans le cas du syndrôme myéloprolifératif induit par le MPSV, la prolifération des cellules néoplasiques dans la rate présentait les mêmes caractéristiques cinétiques que les cellules leucémiques de Friend : coefficient de prolifération cellulaire élevé, durée de cycle mitotique courte ; perte cellulaire importante (90%) dès la phase initiale de la maladie, cette perte étant dûe d'une part à une mort massive *in situ* des cellules proliférantes, et d'autre part à leur différenciation en granulocytes et en érythrocytes.

Cependant, une perte cellulaire massive dès la phase initiale de la maladie, n'est pas une caractéristique commune à l'ensemble des néoplasies murines viro-induites, puisque, dans le cas du sarcome induit par le M-MSV, elle ne dépasse pas 45%. Nos résultats ont également montré que, dans le cas de ce sarcome, l'^{125}IUdR était très largement réutilisé et ne pouvait donc permettre une étude de la mort cellulaire.

En conclusion, nos études ont permis de montrer l'intérêt de l'étude de la cinétique de la prolifération cellulaire pour une compréhension de la physiopathologie des néoplasies expérimentales. Le meilleur exemple en est la leucémie de Friend, où ces études, associées à la connaissance que nous avions des perturbation de l'érythropoièse dans cette maladie nous ont permis de proposer un modèle cohérent de cette néoplasie.

AVANT-PROPOS

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INTRODUCTION

1. BUTS DE L'ETUDE

Il est possible d'induire des leucémies par des virus, chez plusieurs espèces animales (oiseaux, rongeurs, mammifères). Chez la souris, les leucémies ainsi obtenues sont généralement lymphoïdes ; il est également possible d'y provoquer, mais de façon irrégulière, des leucémies myéloïdes aigues ; enfin, plusieurs leucémies érythroïdes ont pû être obtenues. Ces leucémies, dont les caractéristiques sont réunies dans le tableau 1, n'ont cependant pas été considérées, généralement, comme pouvant être des modèles pour certaines leucémies humaines, et l'étude de leur physiopathologie a donc été négligée. Deux leucémies font exception : la leucémie lymphoïde (qui s'apparente à un lymphosarcome), apparaissant spontanément chez les souris de la souche AKR (1) et dûe au virus de Gross, et la leucémie de Friend (7). Cette dernière a fait l'objet d'études particulièrement nombreuses, probablement parce qu'elle représentait un exemple de transformation maligne de la lignée érythroïde. L'érythropoièse est en effet le processus de différenciation hématopoiétique le mieux connu, car son étude a été facilitée par : 1) l'existence d'un marqueur spécifique de l'hémoglobine (^{59}Fe) (12) ; 2) la possibilité de supprimer de façon réversible l'érythropoièse grâce à l'injection de globules rouges (polyglobulie) (13,14,15) ; 3) la connaissance du facteur humoral induisant spécifiquement la différenciation et la prolifération des cellules érythroïdes, l'érythropoiétine (13, 16).

Lorsque nous avons entrepris l'étude de la cinétique de la

TABLEAU 1 : HEMOPATHIES MURINES INDUITES PAR DES RETROVIRUS.

LEUCEMIE	CYTOLOGIE DES CELLULES PATHOLOGIQUES	LATENCE	LOCALISATION DE LA PROLIFERATION PATHOLOGIQUE	OBSERVATIONS	TRANSPLANTABILITE	REFERENCES
Gross (spontanée)	Lymphoïde	5-8 mois	Thymus, ganglion, rate, foie	- la moelle osseuse et le sang sont peu envahis - le rôle du thymus est essentiel - la thymectomie réduit l'incidence des lymphomes	+ + +	1
Moloney		3-4 mois				2
Kaplan (radio-induit) Abelson		3-6 mois				3 4
Graffi	Myeloblastique	2-4 mois	Moelle osseuse, sang circulant, rate, foie	Grande latence entre l'injection du virus et l'apparition de la maladie	+	5
Mc Garry					+ +	6
Friend	Erythroblastique	quelques jours	rate, foie puis sang rein et poumons		0 (exceptionnelle)	7
Rauscher	Erythroblastique puis parfois myéloïde ou lymphoïde	quelques jours 6-8 semaines	rate Moelle osseuse		0 (exceptionnelle)	8
Kirsten	Erythroblastique		rate			9
MPSV	Erythroblastique et myeloblastique	quelques jours	rate, foie, thymus poumons, rein, membrane, péritonéale	Myélofibrose	± (Régression)	10-11

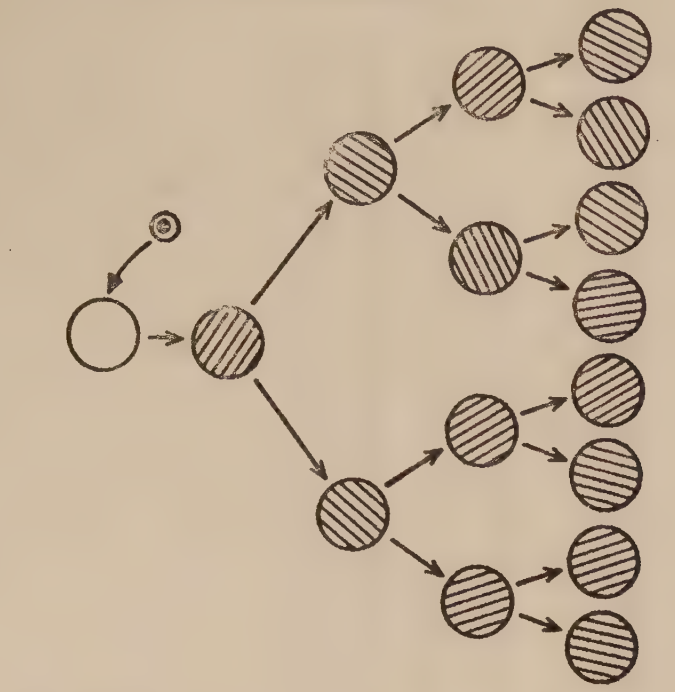
prolifération cellulaire de la leucémie de Friend, il avait déjà été montré que c'était une leucémie érythroblastique dans laquelle la rate et le foie étaient très hypertrophiés et le sang circulant envahi de cellules leucémiques (7,17,18,19,20,21,22).Cependant, on pouvait se demander si l'envahissement des organes hématopoiétiques par les cellules leucémiques résultait d'une prolifération rapide de ces cellules ou, au contraire, de l'accumulation de cellules ne proliférant que très lentement ou pas du tout (Figure 1). Cette seconde hypothèse était en effet plausible car la greffe sous-cutanée de cellules leucémiques à des souris isogéniques était très difficile (7,23,24 25). Une étude de la cinétique de la prolifération des cellules leucémiques apparaissait donc nécessaire à une compréhension de l'ensemble de la maladie.

Les résultats que nous avons obtenus ont mis en évidence l'existence d'un phénomène que les études précédentes n'avaient pas permis de soupçonner, à savoir une perte cellulaire massive, pendant la phase de croissance rapide de la rate, accompagnée d'une mort *in situ* de près des deux tiers des cellules proliférantes.

Ces résultats étaient très différents de ceux obtenus par différentes équipes dans le cas de leucémies qui n'étaient pas induites mais transplantées (26 à 32). C'est pourquoi nous avons étendu nos études à d'autres néoplasies viro-induites, afin de déterminer si une perte cellulaire massive était une caractéristique commune à l'ensemble des néoplasies induites par les rétrovirus, groupe viral auquel appartient le virus de Friend(33).

Figure 1 : MODELE PROLIFERATIF ET CUMULATIF DE LA LEUCEMIE DE FRIEND

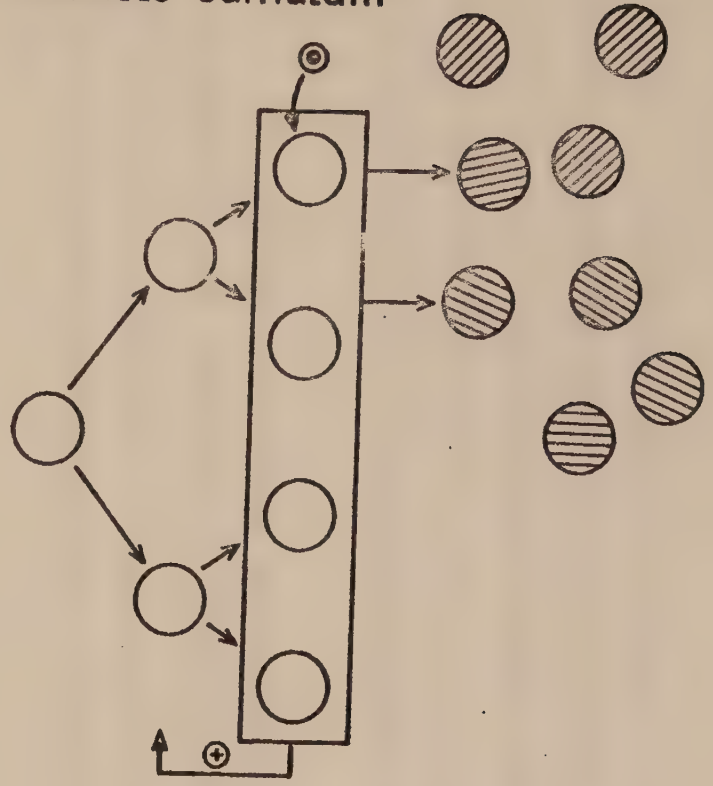
Modèle prolifératif



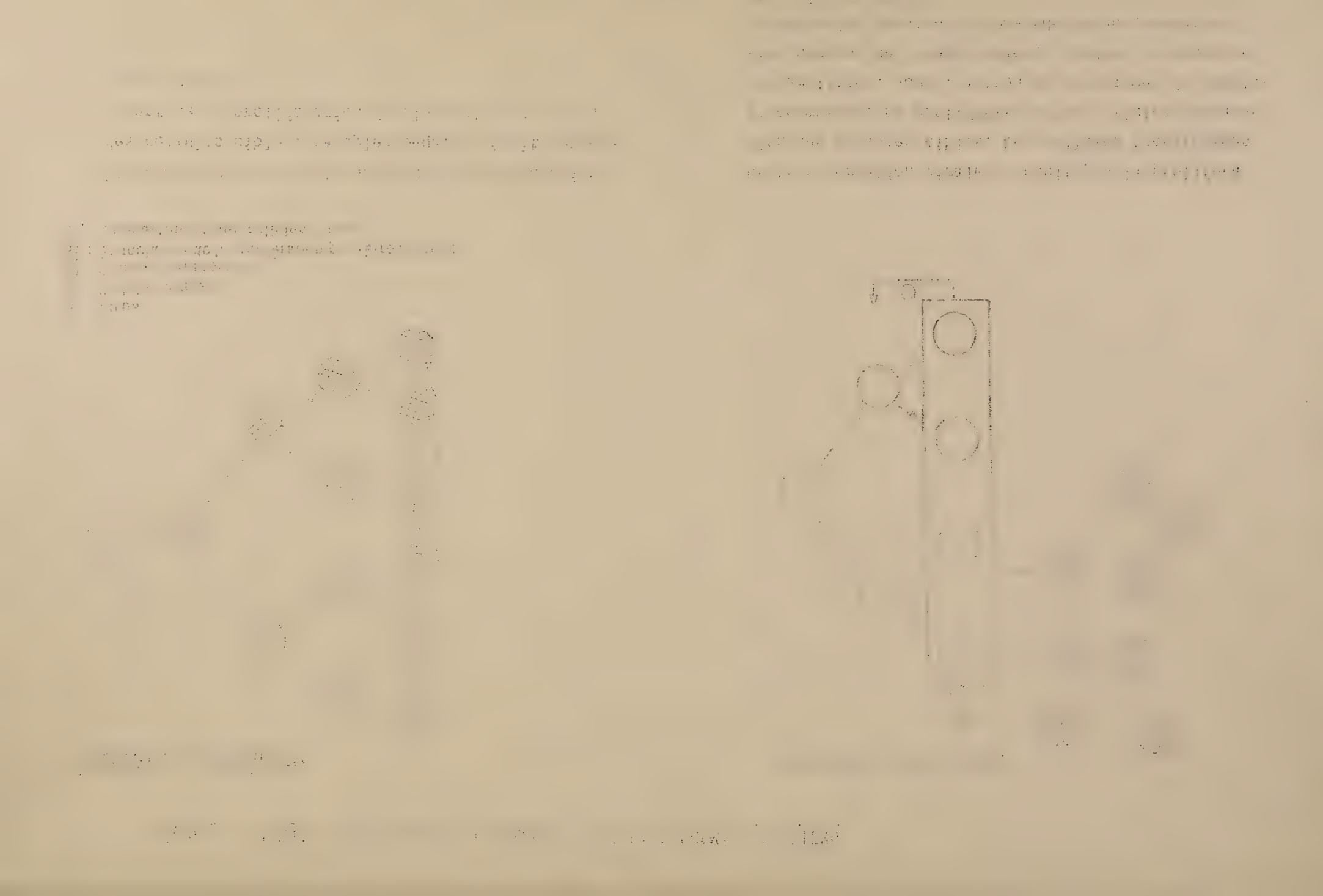
Virus
Cellule normale
Cellule transformée
Stimulation de la prolifération par rétro controle
Compartiment des cellules cibles

La transformation virale provoque la proliferation des cellules cibles. La splénomégalie résulte directement de la prolifération de clone(s) de cellules transformées.

Modèle cumulatif



La transformation virale n'induit pas la prolifération des cellules cibles. Les cellules transformées s'accumulent. La prolifération des cellules normales est amplifiée, sous l'action de mécanismes de régulation normaux de l'hématopoièse, tendant à compenser la déplation constante viro-induite du compartiment des cellules cibles.



Nous avons donc choisi d'étudier la cinétique de la prolifération cellulaire de deux autres néoplasies viro-induites : l'une est hématopoiétique, c'est le syndrôme myéloprolifératif induit par le MPSV (Myeloproliferative Sarcoma Virus (10-11), l'autre est une tumeur solide, c'est le sarcome induit par le Virus Sarcomatogène de Moloney (34).

Comme nous le verrons ultérieurement, nos résultats indiquent que le virus n'est pas directement responsable de cette mort cellulaire rapide des cellules néoplasiques. Ces résultats sont cependant d'une grande utilité pour une meilleure compréhension de la physiopathologie de ces néoplasies.

Dans la suite de cette introduction, nous rappelerons brièvement le modèle actuellement admis de l'hématopoièse normale. Nous résumerons ensuite les principales données concernant la physiopathologie des trois néoplasies que nous avons étudiées. Enfin, nous rappelerons les concepts et les techniques qui sont à la base de l'étude de la cinétique de la prolifération des populations de cellules.

2. L'HEMATOPOIESE NORMALE : CONCEPTS ET DEFINITIONS

Il est habituel de distinguer trois catégories parmi les cellules du sang circulant : les globules rouges -ou érythrocytes-, les plaquettes -ou thrombocytes- et les globules blancs. Par "globules blancs" on désigne en fait plusieurs populations cellulaires distinctes : les lymphocytes,

les granulocytes ou polynucléaires neutrophiles, les éosinophiles, les basophiles et les macrophages. Ces cellules sont différenciées : elles sont spécialisées dans la réalisation d'une fonction (par exemple la fixation d'oxygène ou la phagocytose) et elles ne peuvent plus se diviser (à l'exception toutefois des lymphocytes sous l'action de certains mitogènes (35, 36).

Une hématopoièse continue permet de remplacer les cellules différenciées qui meurent et de maintenir constant leur nombre dans le sang circulant et dans les tissus irrigués (35).

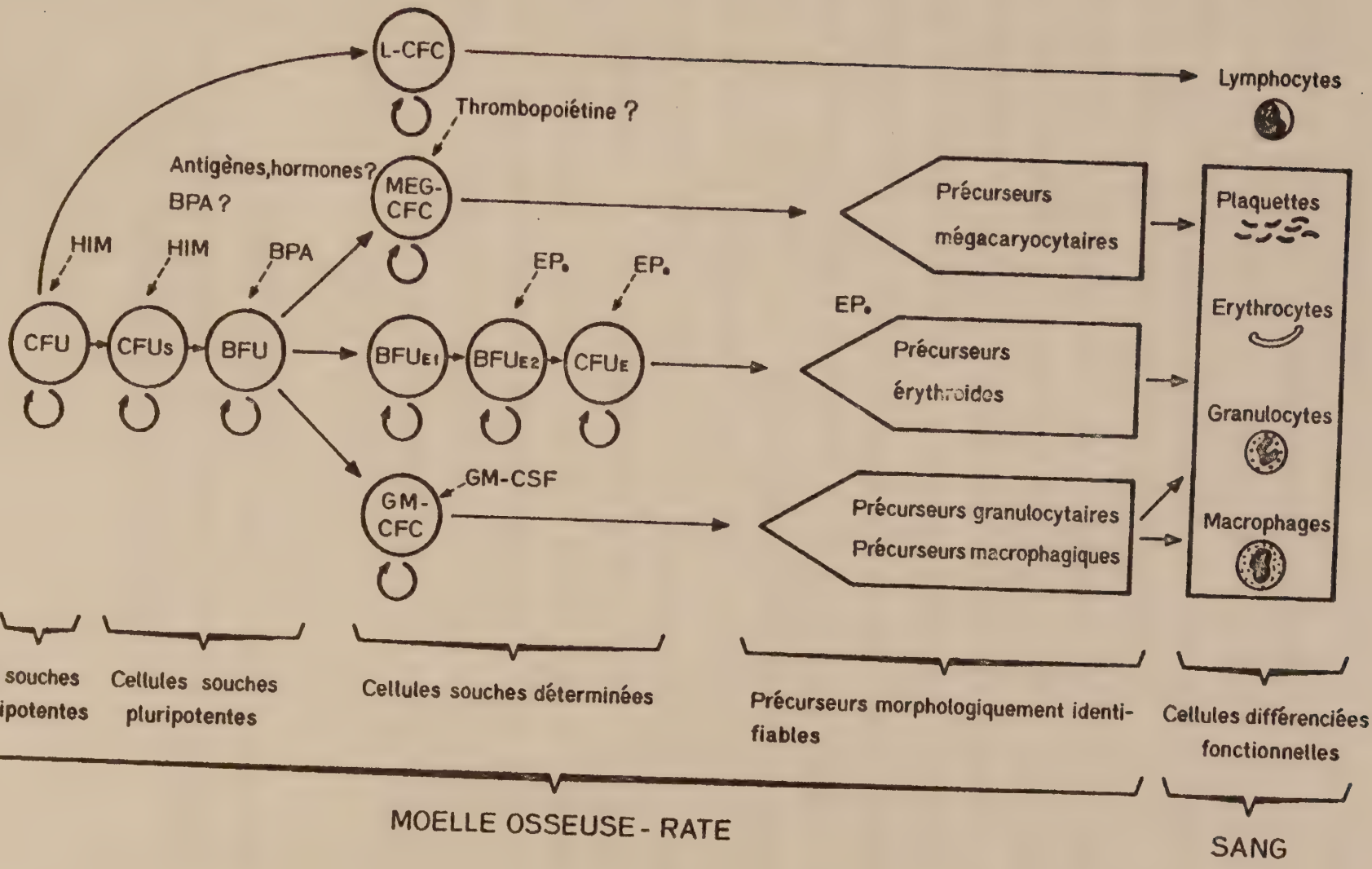
Les cellules hématopoiétiques précurseurs peuvent être groupées en 4 compartiments principaux, chacun d'eux représentant un stade de la différenciation hématopoiétique (Figure 2).

Le compartiment le plus ancestral est celui des cellules souches totipotentes (37) . On ne dispose pas actuellement de méthode expérimentale permettant de les quantifier. Ces cellules sont capables d'une part de s'autorenouveler, c'est-à-dire de donner naissance à des cellules identiques à elles-mêmes, d'autre part, de générer des cellules pluripotentes, qui constituent le second compartiment. Les cellules souches pluripotentes sont dénommées CFUs : "Colony Forming Units in the Spleen". Elles ont en effet la propriété, lorsqu'elles sont injectées par voie intraveineuse à des souris irradiées léthalement, de donner naissance à des colonies visibles à la surface de la rate. Cette propriété permet de les quantifier *in vivo* (38, 39).

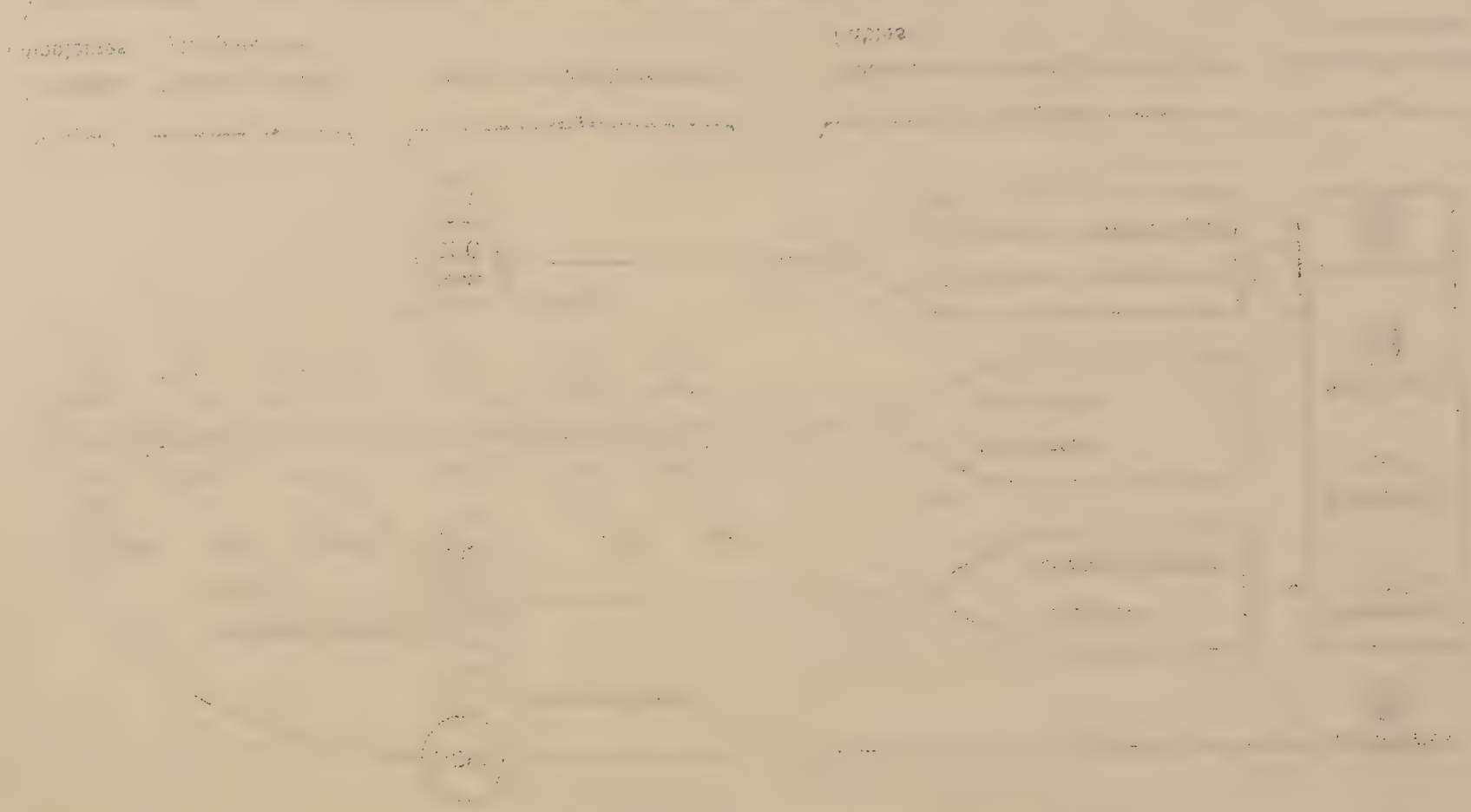
Tableau 2 : Noms et abbréviations des cellules souches hématopoiétiques et des facteurs de contrôle (d'après D. Metcalf 1977)

CELLULES			FACTEURS DE CONTROLE		
Cellules souches	Abbréviations	Synonymes	Nom	Abbréviations	Synonymes
pluripotentes et totipotentes	CFU HSC CFUs	- cellules souches totipotentes Spleen colony forming Units Cellules souches pluripotentes	Facteurs de régulation du microenvironnement	HIM	Hemopoietic inductive microenvironnement
de la lignée granulocytaire et macrophagique	GM - CFC	- Granulocyte - Macrophages. Colony Forming cells - Colony Forming Units Culture CFUc - Colony Forming Units Agar : CFU_A	Granulocyte Macrophage Colony Stimulating Factor	GM - CSF	- Colony Stimulating activity CSF
des macrophages (de type péritonéal)	M - CFC	Macrophage Colony Forming Cells	"	GM - CSF	
des éosinophiles	Eo - CFC	Eosinophil Colony Forming cells	Eosinophil Colony stimulating factor	Eo - CSF	
des mégacaryocytes	MEG - CFC	Megacaryocyte Colony Forming Cells	Megakaryocyte Colony Stimulating factor	MEG - CSF	? Thrombopoiétine
des érythrocytes	BFU_E CFU_E	Burst Forming Units erythroïdes Colony Forming Units erythroïdes	Burst Promoting Activity Erythropoiétine	BPA EPO	
des lymphocytes B des lymphocytes T	BL - CFC TL - CFC	B-Lymphocytes Colony Forming Cells T " " "	? ?	? ?	? ?

Figure 2 : MODELE DE L'HEMATOPOIESE



Abbréviations : Voir Tableau 2



Le troisième compartiment est celui des "cellules souches déterminées" : leur capacité de s'autorenouveler est beaucoup plus restreinte que dans le cas des CFUs. Elles n'ont plus que la propriété de générer une ou deux catégories de cellules hématopoiétiques. Elles sont détectables et quantifiables grâce à leur capacité de donner naissance, *in vitro*, en milieu semi-solide, à des colonies de cellules appartenant à une seule lignée hématopoiétique (40 à 54), ou au maximum à deux d'entre elles : granulocytaires et érythroïdes(55) ou erythroïdes et mégacarypocytaires (56). Cependant, dans le cas de la lignée érythroïde, c'est d'abord *in vivo* qu'ont pu être identifiées des cellules souches déterminées spécifiquement vers la différenciation érythroïde et dont la prolifération et la différenciation étaient spécifiquement sensibles à l'érythropoiétine. Il s'agit des ERC : Erythropoietin Responsive Cells (57,58,59).Il a été ultérieurement montré, grâce aux cultures *in vitro* en milieu semi-solide, que les ERC sont en fait constituées d'au moins 2 catégories cellulaires : les BFU_E (Burst Forming Units erythroid) et les CFU_E (Colony Forming Units erythroid) (60, 61) : les BFU_E sont des précurseurs des CFU_E. Leur prolifération et leur différenciation *in vitro* nécessite la présence de plus grandes quantités d'érythropoiétine que les CFU_E, et est sensible à l'action d'un facteur secrété notamment par les lymphocytes, le BPA (Burst Promoting Activity) (.55, 60, 62).

Les cellules souches "déterminées" donnent naissance, *in vivo*, à des cellules blastiques, les premières à être morphologiquement identifiables et qui constituent le quatrième compartiment (36).

Les cellules hématopoiétiques morphologiquement identifiables ne sont plus capables de s'autorenouveler. Leurs divisions successives s'accompagnent d'une différenciation et de la perte progressive de leur capacité de division. Des critères morphologiques permettent de reconnaître les cellules aux stades successifs de cette différenciation. Dans le cas des différenciations granulocytaire et érythroïde , il est habituel de distinguer le compartiment "prolifératif", qui groupe les cellules ayant encore la capacité de se multiplier, du compartiment de "maturation", qui groupe les cellules ayant perdu cette capacité. La multiplication des cellules blastiques aboutit à la production d'une population accrue de cellules dans le sang circulant (35).

A l'état normal, les quatre compartiments sont en état d'équilibre : vitesse de prolifération et taux de différenciation des cellules sont constants. Cet équilibre est assuré par un système de régulation complexe, faisant intervenir des facteurs de nature différente (Tableau 2, Figure 2). On connaît actuellement :

1) les facteurs du microenvironnement, ou "short-range factors" , facteurs agissant à faible distance du lieu où ils sont produits. Ils participent notamment au contrôle de la différenciation et de la prolifération des cellules souches pluripotentes (63, 64, 65, 66).

2) les facteurs humoraux, ou "long-range factors", qui, au contraire agissent loin de leur lieu de secrétion. Il a

été démontré qu'ils jouent un rôle dans le contrôle de la différenciation et de la prolifération des cellules souches pluripotentes (67,68,69) et surtout dans celle des "cellules souches déterminées" et de précurseurs blastiques morphologiquement identifiables (70,71,72). On distingue généralement les facteurs stimulants, ou poiétines (par exemple : l'érythropoiétine) (73) des facteurs inhibiteurs (par exemple : les chalones) (74). Les facteurs connus actuellement présentent une spécificité pour les cellules d'une des lignées hématopoiétiques (70, 71, 72, 73, 74).

Si l'équilibre entre les différents compartiments du système hématopoiétique est perturbé par un facteur externe, ce système de régulation permet de le rétablir, par stimulation, ou par inhibition de la prolifération et de la différenciation cellulaire (35). Dans certains cas cependant, par exemple après infection par un virus leucémogène, le rétablissement de cet équilibre devient impossible.

Chez la souris, l'hématopoièse se déroule dans la moelle osseuse et dans la rate (35). Dans les modèles expérimentaux que nous avons étudiés, c'est surtout dans la rate qu'est localisée la prolifération des cellules leucémiques : la structure de cet organe a été représentée dans la Figure 3.

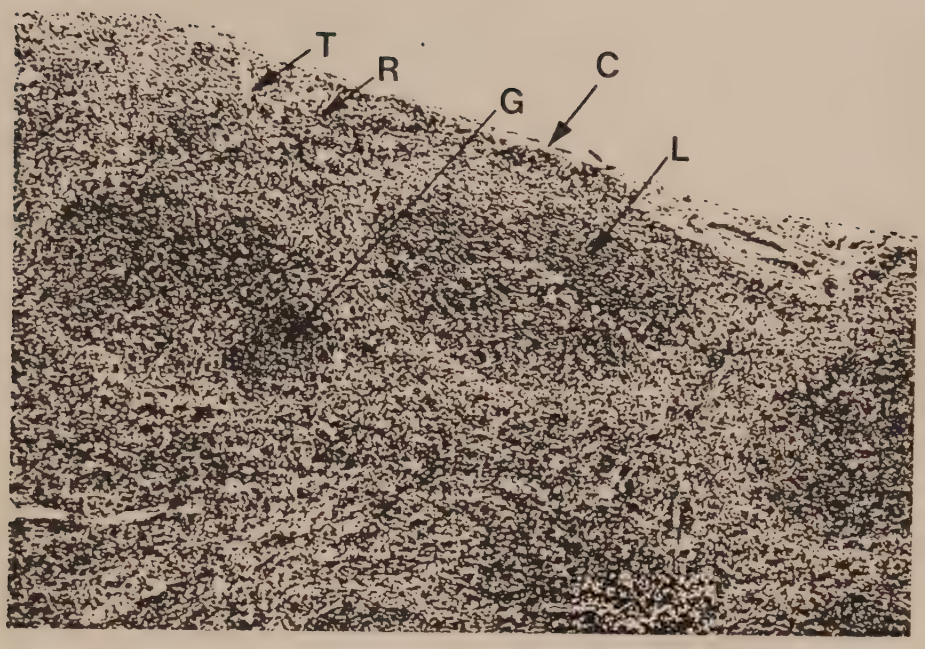


Figure 3 : Rate de souris adulte

Photographie d'une rate de souris, montrant plusieurs follicules lymphoïdes (L) avec leurs centres germinatifs (G) pulpe rouge (R) et les trabécules fibreuses (T) hématoxyline-éosine x25. Les lymphocytes sont concentrés dans les follicules lymphoïdes (sphériques ou ovoïdes), au centre desquels on observe une artériole centrolobulaire. Le tissu érythroïde est réparti en amas à travers la pulpe rouge dans laquelle on observe quelques mégakaryocytes. Les cellules granulocytaires sont préférentiellement localisées à la périphérie de la pulpe rouge, sous la capsule (C) et le long des trabécules fibreuses.

Toutes les cellules entrent ou sortent de cet organe par le sang.

3. CARACTERISTIQUES PRINCIPALES DES TROIS NEOPLASIES VIRALES ETUDIEES

3.1. La leucémie de Friend

L'injection du virus de Friend à des souris de souche sensible provoque rapidement une hépatosplénomégalie, le poids de la rate pouvant être multiplié par un facteur 30 environ, un mois après l'injection. Une érythroblastose, associée à une légère lymphocytose et à une thrombocytopénie, est observée dans le sang circulant. Deux virus Friend sont actuellement connus, l'un provoquant une anémie (c'est de VFA : virus de Friend anémiant) (7), l'autre une polyglobulie (c'est le VFP : virus de Friend polycythémiant) (19,21,75).Le déroulement de la maladie ne semble pas être influencé par le thymus qui, comme les ganglions lymphatiques, n'est pas modifié après l'infection virale (7). Il a été montré que les premières cellules leucémiques -dites aussi cellules hyperbasophiles ou cellules de Friend- apparaissent dans la rate, dès 30-40 heures après l'injection du virus (22). Elles sont alors observées uniquement dans la pulpe rouge splénique, et en dehors des zones granulopoiétiques et mégacaryocytaires. Ces cellules hyperbasophiles ont les caractéristiques cytologiques de proérythroblastes jeunes (17, 18)et peuvent se différencier en érythrocytes (20, 22). Bien qu'il ait été démontré que le nombre des cellules hématopoiétiques des différentes catégories des cellules souches (CFUs et GM-CFC) soit augmenté au cours de la maladie (76, 77, 78), le VFP ne transforme spécifiquement qu'une catégorie de cellules souches, celles déterminées vers la différenciation érythroide, les ERC (21,22,79,80,81) . La transformation de ces cellules cibles s'exprime par

leur différenciation, indépendante de l'érythropoiétine, en cellules hyperbasophiles. Les études *in vitro* et *in vivo* ont également montré que les CFU_E, spléniques et médullaires, sont augmentées et capables de produire *in vitro* des colonies érythroïdes en l'absence d'érythropoiétine (82,83,84,85,86). Enfin, les cellules leucémiques de Friend ne peuvent pas être aisément transplantées par la méthode classique de la greffe sous-cutanée à des souris isogéniques (7, 23,24,25,87,88). Ces caractéristiques sont résumées dans le tableau 3.

L'ensemble de ces données physiopathologiques, et plus particulièrement l'impossibilité de greffer facilement les cellules hyperbasophiles de Friend conduisait donc à s'interroger sur la capacité de ces cellules à se multiplier. Nous avons donc entrepris d'étudier la cinétique de la prolifération cellulaire dans la leucémie de Friend. La leucémie induite par le VFP nous a semblé être un meilleur modèle pour cette étude que la leucémie induite par le VFA. En effet, la rate n'y est pas hémorragique, et l'augmentation du nombre de cellules leucémiques y est donc plus simple à mesurer.

3.2. Le syndrôme myéloprolifératif induit par le MPSV

Un virus capable d'induire rapidement et de façon reproductible une splénomégalie lorsqu'il était injecté à des souris adultes, fut isolé par M.A. Chirigos en 1968, à partir du plasma de souris adultes infectées par le virus sarcomatogène de Moloney (MSV). Cette splénomégalie fut principalement attribuée à une prolifération néoplasique des cellules mésenchymales de la rate (11).

Tableau 3 : PRINCIPALES PERTURBATIONS DE L'HEMATOPOIESE PAR LES VIRUS VFA ET VFP CHEZ LES SOURIS DBA_2 OU C3H.

Site anatomique	Pathologie	CFUs		GM-CFC		CSF-GM-CFC		CFU_E		CFU_EEP indépendants	
		VFA	VFP	VFA	VFP	VFA	VFP	VFA	VFP	VFA	VFP
Moelle osseuse	. Envahissement tardif par CL	Normal	Normal	NR	NR	ND	ND	x 2	x 20	ND	OUI
Rate	. Poids x 30 . envahissement précoce par CL	x 15.20 C normale	x 15.20 C normale	x 300	NR	ND	ND	x 40	x 40	ND	OUI
Sang circulant	. Envahissement progressif par CL . Lymphocytose . Thrombopenie VFP : polyglobulie VFA : anémie	NR	x 800	NR	NR	NR	NR	NR	NR	NR	NR

ND : non détecté

NR : non recherché

CL : cellules leucémiques de Friend, hyperbasophiles

Les chiffres indiquent l'évolution du nombre absolu de cellules souches dans les différents organes considérés

CFUs, GM-CFC, CFU_E, CSF, EP : voir définitions Tableau 2

CSF^-GM-CFC : GM-CFC proliférant *in vitro* sans addition de CSF

ASPECT DE LA RATE DE SOURIS ADULTE NORMALE OU INFECTEE PAR LES VIRUS VFP OU MPSV

De gauche à droite :

- rate normale, pesant environ 80 mg
- rate de souris présentant la leucémie de Friend induite par le VFP. C'est une rate rouge, érythroblastique.
- rate de souris présentant le syndrôme myéloprolifératif induit par le MPSV. C'est une rate blanche, envahie de nodules tumoraux.

Les rates néoplasiques ont été prélevées trois semaines après l'infection virale et elles pèsent environ 1800 mg.

Dans notre laboratoire, nous avons observé chez les souris de souche DBA_2 infectées par ce virus, d'importantes perturbations hématologiques, dont l'ensemble permet de caractériser généralement les syndrômes myéloprolifératifs humains (89,90): métaplasie granulocytaire et érythroïde dans la rate, dont le poids est très augmenté, et dans le foie ; anémie, augmentation variable du réseau de réticuline dans la moelle osseuse (myélofibrose). C'est pourquoi nous avons appelé ce virus "MPSV" : Myeloproliferative Sarcoma Virus. Une nouvelle dénomination étant d'autant plus nécessaire que le virus original qui nous avait été donné par M.A. Chirigos pouvait avoir été modifié après s'être répliqué dans les souris de notre laboratoire.

L'injection du MPSV aux souris DBA_2 provoque rapidement une hyperplasie de la pulpe rouge splénique, une prolifération des cellules érythroïdes et granulocytaires dans la rate, une métaplasie hématopoiétique dans le foie, l'apparition de cellules blastiques dans le sang circulant et une fibrose dans la moelle osseuse, la rate et le foie. On observe de plus un phénomène jusqu'alors non décrit dans les néoplasies expérimentales, à savoir une diminution rapide et importante du poids de la rate et du foie peu de jours avant la mort des souris infectées. Ce phénomène est concommittant avec une augmentation importante du nombre de cellules érythroïdes et granulocytaires dans le sang circulant (10).

A ce syndrôme myéloprolifératif typique s'ajoute l'existence de nodules d'aspect tumoral : ce sont des structures bien individualisées histologiquement, observées dans les organes hématopoiétiques : rate, thymus,

foie, ganglions, et non hématopoiétiques : muscles, poumons, reins, organes génitaux et membrane péritonéale, sur laquelle ils sont macroscopiquement visibles. Ces nodules sont constitués par l'association de 3 types cellulaires principaux présents en proportions égales : de grandes cellules blastiques, qui ne peuvent être colorées par les colorations cytochimiques connues, des cellules granulocytaires et des cellules d'aspect fibroblastique. La rate est le premier organe dans lesquels ils sont observés ; ils y apparaissent autour des artérioles centrolobulaires des follicules lymphoïdes puis envahissent progressivement tout l'organe (10).

Les études physiopathologiques qui ont été effectuées dans notre laboratoire ont montré que le nombre des cellules souches hématopoiétiques CFUs et GM-CFC était très augmenté dans la rate néoplasique (x 110), leur augmentation résultatnt à la fois de celle de l'ensemble de la population splénique (x 11) et de leur concentration (x 10). Les CFUs, ainsi que les GM-CFC conservent une capacité de maturation normale. Cependant, le MPSV induit des modifications qualitatives importantes de GM-CFC : en effet, une proportion variable des GM-CFC spléniques et médullaires (de 10% à 60% selon les souris) a la capacité de donner naissance à des colonies macrophagiques et granulocytaire *in vitro* sans qu'il soit nécessiare d'ajouter du CSF (91)..

Des clônes viraux ont été obtenus, qui induisent la même maladie que le virus non clôné. Les virus clonés sont constitués de deux entités distinctes:

- un virus helper de la réplication virale
- un virus sarcomatogène dont le génome code deux fonctions

biologiques distinctes : la transformation *in vitro* de fibroblastes embryonnaires de souris et l'induction de foyers de cellules "transformées", *in vivo*, sur la rate de souris de souche sensible. Ce génome, bien que plus grand que celui des virus sarcomatogène murins (MSV) défectifs connus jusqu'à présent (7000 paires de bases au lieu de 5000 environ), ne possèderait cependant pas de séquences qualitativement différentes de celles des MSV (92).

Les principales caractéristiques physiopathologiques de la néoplasie induite par le MPSV sont résumées dans le Tableau 4. Nous avons étudié la cinétique de la prolifération cellulaire de cette néoplasie car nous désirons déterminer si, comme dans les leucémies de Friend et de Rauscher (93, 94, 95) les cellules de la rate néoplasique seraient caractérisées par un taux de renouvellement particulièrement rapide, ou si, au contraire, leur prolifération serait plus lente et affectée d'un taux de perte cellulaire plus faible comme dans la plupart des tumeurs solides (96). En effet, bien que, comme le virus de Friend, le MPSV induise une augmentation importante du nombre de cellules hématopoiétiques, la néoplasie provoquée par le MPSV évoque aussi une tumeur solide : la rate est dure et blanche, et des nodules tumoraux sont observés, notamment sur le péritoine. Au contraire, dans la leucémie de Friend, la rate reste très rouge, et on n'observe pas de nodules tumoraux. D'autre part, comme dans la leucémie de Friend, une quantification de la prolifération cellulaire nous semblait être nécessaire à une compréhension de l'ensemble du processus pathologique.

Tableau 4 : PRINCIPALES PERTURBATIONS DE L'HEMATOPOIESE CHEZ LES SOURIS DBA_2 INFECTEES PAR LE MPSV.

Site anatomique	Pathologie	CFUs	GM-CFC	CSF⁻GM-CFC	CFU_E	Remarques
Moelle osseuse	- myelofibrose	x 0.5 x 0.5	x 0.5 x 0.5	oui oui en fin de maladie	x 3 x 3	ND ND
Rate	- hyperplasie de la pulpe rouge - Nodules tumoraux - Poids x 20 - fibrose	x 100 concentration x 10	x 100 concentration x 10	oui (10 à 60%)	x 40	hypersensibilité des CFU_E à l'EP.
Sang circulant	- Anémie - Thrombopénie - Erythroblastose - myeloblastose	x 100	x 150 concentration x 14	oui en fin de maladie	NR	NR
Membrane péritonéale, foie, coeur, poumon, reins.	Nodules tumoraux constitués de myeloblastes, fibroblastes, blastes indifférenciés	NR	NR	NR	NR	NR

ND : non détecté

NR : non recherché

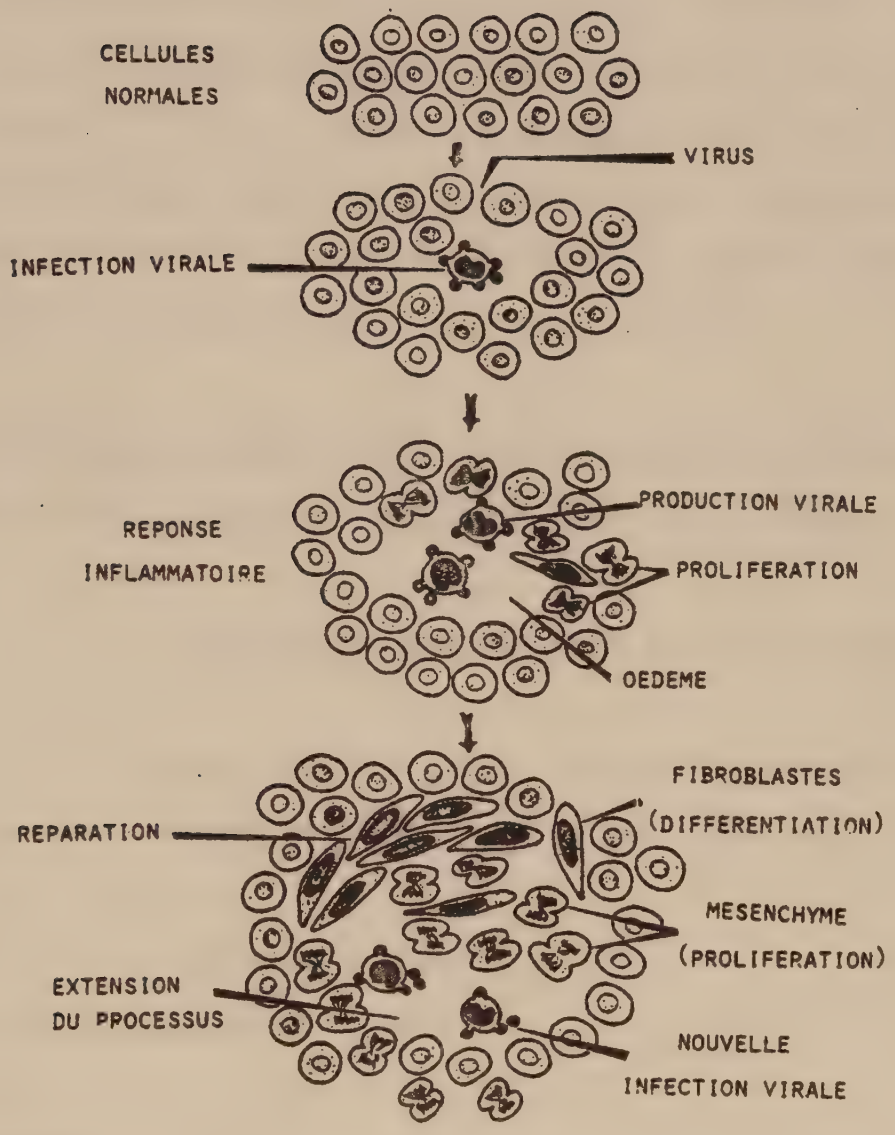
CFUs, GM-CFC, CFU_E, CSF, EP : voir définitions Tableau 2

3.3. Le sarcome induit par le virus de Moloney (Sarcome de Moloney)

Le sarcome de Moloney représente un modèle de néoplasie induite par un rétrovirus, comme les deux hémopathies malignes décrites précédemment, mais dans ce cas la tumeur est solide et les cellules ne sont pas hématopoiétiques.

Moloney isola, en 1966, un virus sarcomatogène (M-MSV) à partir d'un rhabdomyosarcome induit chez les souris BALB/c par l'injection de doses élevées du virus de la leucémie de Moloney (Mu-MLV) (34). L'injection du M-MSV dans le tissu musculaire provoquait *in situ* l'apparition de tumeurs que Perk et Moloney attribuèrent à la prolifération néoplasique de cellules d'origine musculaire (97). Cependant Stanton contesta cette interprétation, parce que ces tumeurs régressaient souvent chez l'adulte et qu'il était difficile de les transplanter. Il les caractérisa au contraire, comme des granulomes contenant des cellules mésenchymales atypiques (98). R. Siegler appuya cette hypothèse par une étude histopathologique qui suggérait que le premier effet de l'infection virale était de provoquer un oedème, qui était ensuite infiltré par des lymphocytes et des macrophages. R. Siegler proposa un modèle de la maladie, que nous avons reproduit dans la Figure 4. Dans ce modèle, la tumeur était le siège d'une destruction cellulaire massive, provoquée par le virus, et la prolifération de cellules fibroblastiques et mésenchymales était considérée comme équivalent à un processus de cicatrisation (99).

Figure 4 : MODELE DU PROCESSUS PATHOLOGIQUE INDUIT *IN VIVO* PAR LE VIRUS SARCOMATOGENE DE MOLONEY (D'après Siegler R. 1968) (99).



Le virus provoque une destruction cellulaire et la formation d'oedème. Ce phénomène provoque la multiplication des cellules normales contigues (réponse inflammatoire). Ce phénomène est entretenu par la replication constante du virus dans les cellules.

L'étude de la prolifération cellulaire dans le sarcome de Moloney avait donc pour but de déterminer si une perte cellulaire massive en partie dûe à une mort cellulaire *in situ* serait observée dès la phase de croissance rapide de cette tumeur solide et non hématopoiétique, comme c'est le cas dans les rates néoplasiques des souris présentant la leucémie de Friend ou le syndrôme myéloprolifératif induit par le MPSV. L'hypothèse selon laquelle, *in vivo*, les rétrovirus provoqueraient la lyse rapide des cellules transformées aurait alors pû être envisagée. Nous désirions également vérifier ainsi, l'hypothèse de Siegler selon laquelle le sarcome de Moloney était le siège d'une mort cellulaire importante.

4. LA CINETIQUE DE LA PROLIFERATION CELLULAIRE DES POPULATIONS EN CROISSANCE EXPONENTIELLE : RESUME DES THEORIES ET TECHNIQUES UTILISEES.

4.1. Définition d'une population en croissance exponentielle

Une population est dite en croissance exponentielle si sa taille suit une courbe exponentielle, définie par l'équation:

$$N = N_o \, e^{kt}$$

N : taille de la population au temps t

N_o: taille de la population au temps 0

t : temps

k : constante

Une telle population présente une croissance asynchrone: en effet, elle peut être considérée comme résultant de la multiplication de cellules ayant des durées de cycle mitotique légèrement différentes.

4.2. Modèles de croissance exponentielle. Le modèle de Mendelsohn

Le concept de prolifération est toujours défini en relation avec un modèle, dans lesquelles les cellules sont définies comme étant proliférantes (P) ou non proliférantes (Q) au moment de leur naissance.

Le modèle de Mendelsohn

Parmi les différents modèles tendant à représenter les processus aboutissant à la croissance d'une population cellulaire, celui élaboré par Mendelsohn en 1960 (100) a été particulièrement important pour les expérimentateurs, et c'est pourquoi nous en rappelerons les principales caractéristiques. Dans ce modèle les hypothèses suivantes sont faites:

a) il y a 2 catégories de cellules, les cellules proliférantes P et les cellules quiescentes (Q) ;

b) c'est à leur naissance et non à n'importe quel moment du cycle cellulaire que les cellules entrent en P ou en Q ;

c) lorsque les cellules sont arrivées en Q elles ne peuvent revenir en P ;

d) il n'y a ni fuite, ni mort cellulaire.

Le concept de cellules quiescentes (Q, ou en G_o) s'appuie sur des arguments expérimentaux (101) et est certainement valable dans la plupart des systèmes biologiques. Par contre, l'hypothèse de l'absence de

fuite et de mort cellulaire n'est généralement pas vérifiée. Cependant, le modèle de Mendelsohn reste applicable si l'on suppose que la fuite ou la mort cellulaire existent mais affectent avec la même probabilité les cellules des compartiments P et Q, ce qui est vraisemblable. Les durées des différentes phases du cycle cellulaire étant connues, le paramètre d'intérêt de ce modèle est le pourcentage des cellules qui sont en P. C'est le pourcentage qu'on appelle le coefficient de prolifération cellulaire ("Growth Fraction" en anglais) (100). Ce modèle, que nous avons représenté schématiquement dans la Figure 5 a eu du succès à cause de sa simplicité, du fait que la notion de cellules quiescentes était résumée par un seul chiffre pour chaque système expérimental et, enfin, parce qu'un procédé expérimental simple de mesure de ce nombre avait été immédiatement proposé par Mendelsohn.

4.3. Principaux paramètres de la cinétique de la prolifération cellulaire

Les principaux paramètres permettant de caractériser la prolifération d'une population de cellules ont été résumés dans le Tableau 5. Nous y avons également indiqué les abbréviations, les synonymes et les méthodes de calcul principalement utilisés. Il nous a cependant semblé utile d'en rappeler les principales définitions (102).

Durée du cycle mitotique : c'est le temps pendant lequel une cellule ou un groupe de cellules accomplissent un cycle de division mitotique.

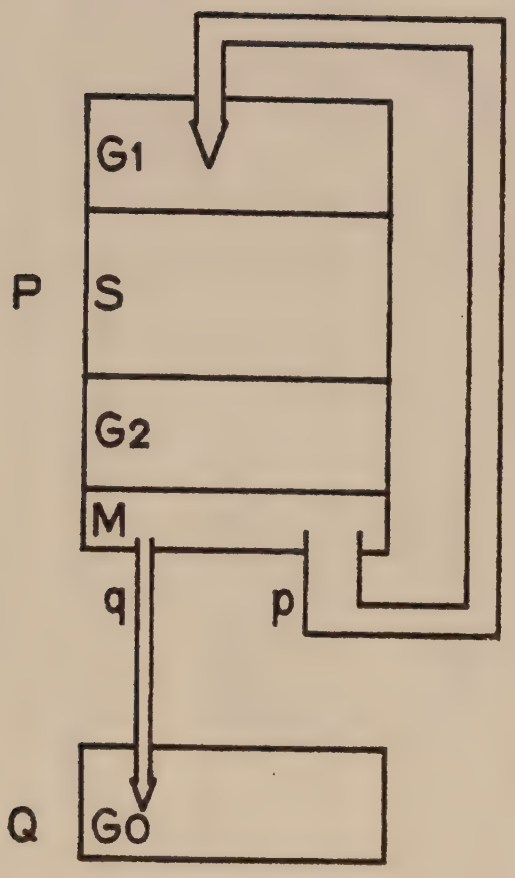


Figure 5 : Modèle de Mendelsohn

C'est un modèle à 2 compartiments : à chaque mitose, les cellules qui viennent de naître se dirigent en proportion p vers P et q = 1 - p vers Q. Le coefficient de prolifération GF est égal à la proportion de cellules en P : $GF = \frac{P}{P+Q}$

Il n'y a pas dans ce modèle de retour de Q vers P.

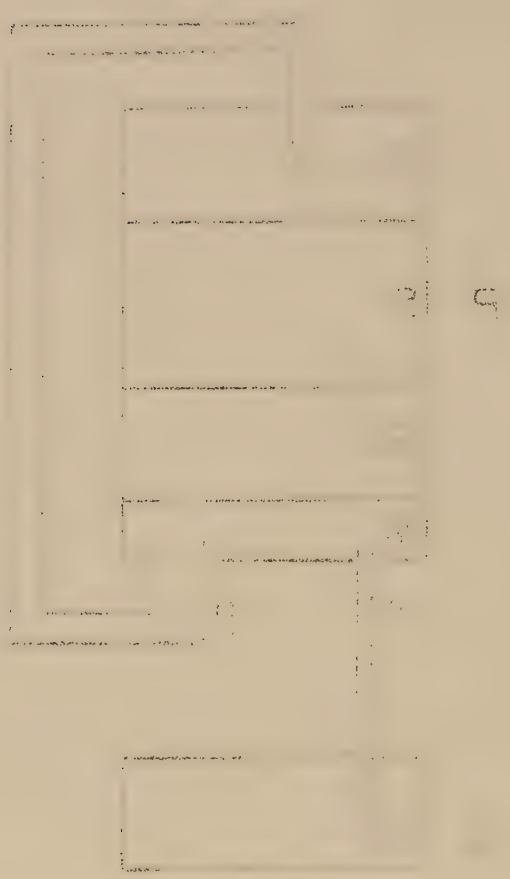


Tableau 5 : Résumé des principaux paramètres de la cinétique de la prolifération des populations cellulaires en croissance exponentielle

Nom	Abbreviation	Synonymes	Méthode de calcul
Durée du cycle cellulaire	Tc	Durée du cycle mitotique T_M	Courbe des mitoses marquées
Durée de la phase de synthèse de	Ts	-	- Courbes de mitoses marquées - Méthode de double marquage isotopique
Index mitotique	MI	Mitotic Index	-
Index de marquage	LI	Labelling Index	Autoradiographie
Vitesse de production cellulaire	k_p	-	$k_p = \frac{\ln (1 + GF)}{Tc}$
Coefficient de la prolifération cellulaire	GF	Growth Fraction	$GF \simeq \frac{LI \exp}{Ts/Tc}$
Temps de doublement réel	TD	- temps de doublement - doubling time DT	- mesure directe de la taille de la population
Temps de doublement potentiel	TD pot.	- Potential Doubling time DT pot. - T pot.	$TD\ pot. = \frac{\ln 2}{kp} \simeq 0.7 \frac{Ts}{LI}$
Vitesse de perte cellulaire	k_1	-	$k_1 = \emptyset \cdot kp = \emptyset \frac{\ln(1 + GF)}{Tc}$
Facteur de perte cellulaire	$\emptyset$	Cell loss factor	$\emptyset = \frac{k_1}{kp} = 1 - \frac{TD\ pot.}{TD}$
Temps de renouvellement	T_t	- Durée du Turn-over	$T_t = \frac{1}{kp}$ Si $GF < 1$, $\emptyset = 0$

Phases du cycle mitotique : parties du cycle mitotique qui peuvent être définies sur des critères morphologiques ou biochimiques. Habituellement, on distingue schématiquement 4 phases successives :

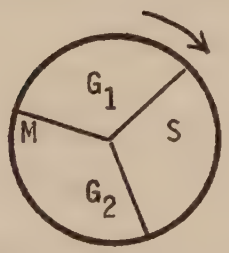
G_1 (GAP-PHASE en anglais) : c'est la phase présynthétique, qui s'étend de la fin de la mitose au début de la phase de synthèse de l'ADN. Le contenu en ADN des cellules est alors égal à N.

S : phase de synthèse de l'ADN.

G_2 : phase post-synthétique. Elle s'étend de la fin de la synthèse de l'ADN jusqu'à la phase de mitose.

M : phase de mitose ou de division cellulaire.

Ces phases se succèdent selon un cycle généralement schématisé comme suit :



la flèche indique l'ordre de succession des différentes phases

Index mitotique : c'est la fraction (ou le pourcentage) de cellules d'une population qui présentent les caractéristiques morphologiques d'une mitose.

Index de marquage : c'est la fraction (ou le pourcentage) de cellules d'une population qui sont rapidement marquées après une injection unique de ^{3}HTdR.

Ce paramètre est mesuré généralement 1 heure après l'injection de ^{3}HTdR et représente habituellement le pourcentage de cellules qui sont en phase S du cycle mitotique. Cependant, ce paramètre est très dépendant de la qualité technique des autoradiogrammes (problème du "Bruit de Fond". (106)).

Coefficient de la prolifération cellulaire ("Growth Fraction" en anglais) : c'est la fraction (ou le pourcentage) de cellules qui sont considérées comme étant proliférantes (100).

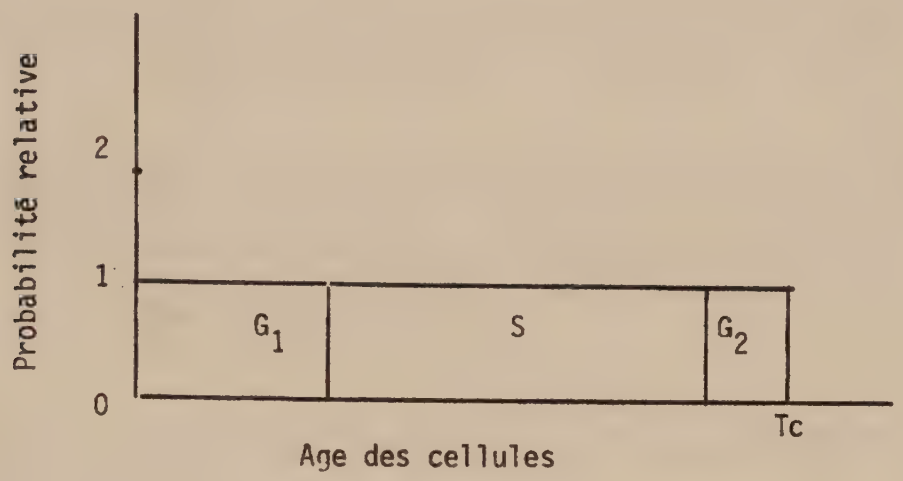
Temps de doublement (réel) d'une population cellulaire : c'est l'intervalle de temps pendant lequel le nombre de cellules d'une population cellulaire en croissance exponentielle est multiplié par 2.

Vitesse de production cellulaire kp : c'est la vitesse à laquelle les cellules sont ajoutées à la population cellulaire, par mitose.

Temps de doublement potentiel d'une population cellulaire : c'est le temps de doublement qui aurait caractérisé une population cellulaire en l'absence de perte cellulaire. On suppose que les cellules qui auraient dû être perdues restent dans la population, sans qu'il y ait modification du coefficient de prolifération cellulaire ou de la distribution d'age (102).

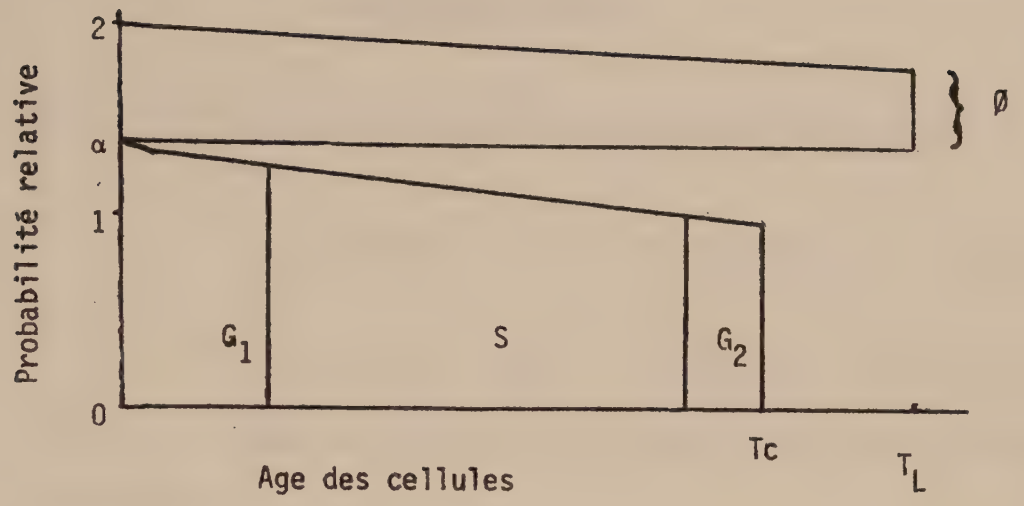
Distribution d'age (ou structure d'âge) d'une population en croissance exponentielle : Définissant l'âge d'une cellule comme le temps écoulé depuis sa nais-

sance, on appelle distribution des âges ou structure d'âge d'une population la répartition des cellules dans les différentes classes d'âge. Cette distribution peut être figurée par une courbe représentant la probabilité relative qu'ont les cellules d'une popualtion d'être dans une des phases du cycle mitotique. Dans le cas d'une population en équilibre, la distribution est "rectangulaire" et peut être représentée de la façon suivante :



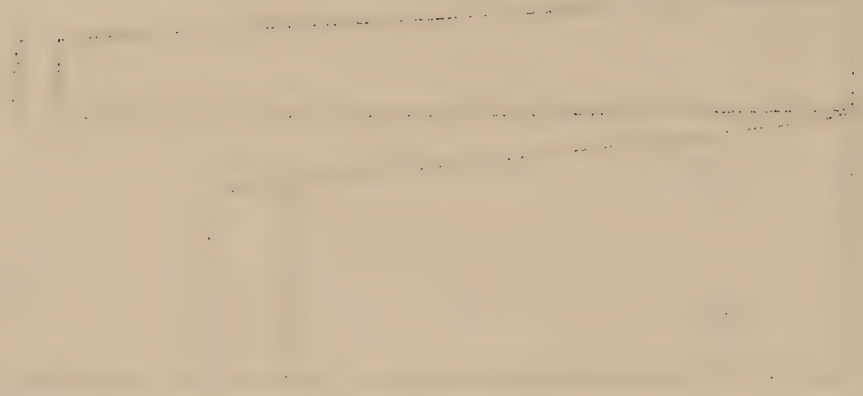
Dans ce cas, la probabilité qu'a une cellule d'être à une phase quelconque du cycle cellulaire est constante.

Au contraire, les cellules de populations en croissance exponentielle, auront une probabilité plus grande d'être en G_1 qu'en S ou en G_2. Le schéma suivant représente un exemple de répartitions d'âge dans une population en croissance exponentielle où seule une partie des cellules prolifère. Dans cet exemple, les cellules non proliférantes peuvent atteindre un âge maximum T_L au delà duquel elles sont perdues. α est le nombre moyen de cellules produites à chaque division qui continuent à proliférer.



Facteur de perte cellulaire : Ce facteur est défini comme étant le rapport du nombre de cellules perdues sur le nombre de cellules produites au sein d'une population cellulaire (103).

Temps de renouvellement (Turn-over time) d'une population : C'est le temps pendant lequel une population cellulaire produirait une quantité de cellules égale à la quantité présente à un instant donné, si la vitesse de production cellulaire reste constante. C'est une notion statistique, qui ne signifie donc pas que chaque cellule de la population a été remplacée. En effet, les cellules d'une population ne se divisent pas de façon synchrone mais, au contraire, sont réparties dans les différentes classes d'âge. (Se reporter au paragraphe concernant la distribution d'âge d'une population).



4.4. Techniques d'étude

4.4.1. Thymidine tritiée et (^{125}I) 5-Iodo-2'-désoxyuridine

Le marquage d'une cellule doit remplir les conditions suivantes :

- la liaison entre le marqueur et la cellule doit être stable : le marqueur doit rester incorporé à la cellules pendant toute la vie de celle-ci.

- Après la mort de la cellule marquée, le marqueur ne doit pas être réincorporé par une autre cellule.

- Le marqueur ne doit pas modifier le comportement de la cellule : il doit être dépourvu de toxicité, immédiate ou différée.

- Les molécules du marqueur non incorporées doivent être rapidement éliminées de l'organisme.

Aucun marqueur ne satisfait de façon parfaite à ces trois exigences, mais certains précurseurs radioactifs tels que la thymidine tritiée ou la ^{125}I-5-iodo-2'-désoxyuridine (^{125}IUdR) de l'acide désoxyribonucléique (ADN) sont ceux qui s'en rapprochent le plus, ce qui explique leur utilisation intensive dans les études de la cinétique de la prolifération cellulaire. En effet, l'ADN dans lequel ces précurseurs s'incorporent spécifiquement est l'un des constituants cellulaires les plus stables. Il est donc possible de considérer que l'évolution de la radioactivité représente l'évolution des cellules qui l'ont incorporée. D'autre part, ces

précurseurs s'incorporent principalement au cours du processus de duplication de l'ADN, processus accompli par les cellules qui vont se diviser. Leur incorporation est donc généralement étroitement corrélée à une activité mitotique (104).

Le précurseur radioactif de l'ADN le plus utilisé est la thymidine tritiée (^{3}HTdR) (104,105).La structure biochimique de cette molécule ainsi que le mécanisme de son incorporation dans l'ADN cellulaire sont représentés dans la Figure 6. Les propriétés des particules émises par l'atome de tritium (^{3}H) sont résumées dans le Tableau 6. Le parcours de ces particules étant très faible, les cellules ayant incorporé la thymidine tritiée pourront être identifiées individuellement, grâce à la technique d'autoradiographie (106).

Cependant, après la mort des cellules marquées, des proportions parfois non négligeables (107, 108) d'^{3}HTdR peuvent être réutilisées par d'autres cellules en prolifération. Ce phénomène limite l'utilisation de cette molécule, notamment dans les études de la perte cellulaire et justifie l'utilisation, dans certains systèmes expérimentaux, d'une autre molécule, la 5-iodo-2'-désoxyuridine (^{125}IUdR) (109 à 115).

L'^{125}IUdR, dont la structure biochimique est représentée dans la Figure 6 est incorporée spécifiquement dans l'ADN cellulaire, mais cette incorporation s'effectue à un taux environ 10 fois plus faible que dans le cas de l'^{3}HTdR. Les propriétés des radiations émises par l'^{125}I, résumées dans le Tableau 6, ont deux conséquences pratiques importantes : une

Tableau 6 : RADIATIONS EMISES PAR LE TRITIUM ^{3}H ET L'IODE 125 (^{125}I)

Isotopes	^{3}H	^{125}I	
Demie-vie	12,26 ans	60 jours	
particules émises	β	rayonnement électro-magnétique (RX et $R\gamma$)	e^- (Auger etc..)
Energie maximum	19 KeV	35 KeV et 60 KeV	30 KeV
Parcours maximum dans l'eau	1,13 μ		environ 1 μ
Parcours maximum dans l'émulsion autoradiographique	0,45 μ		environ 1 μ

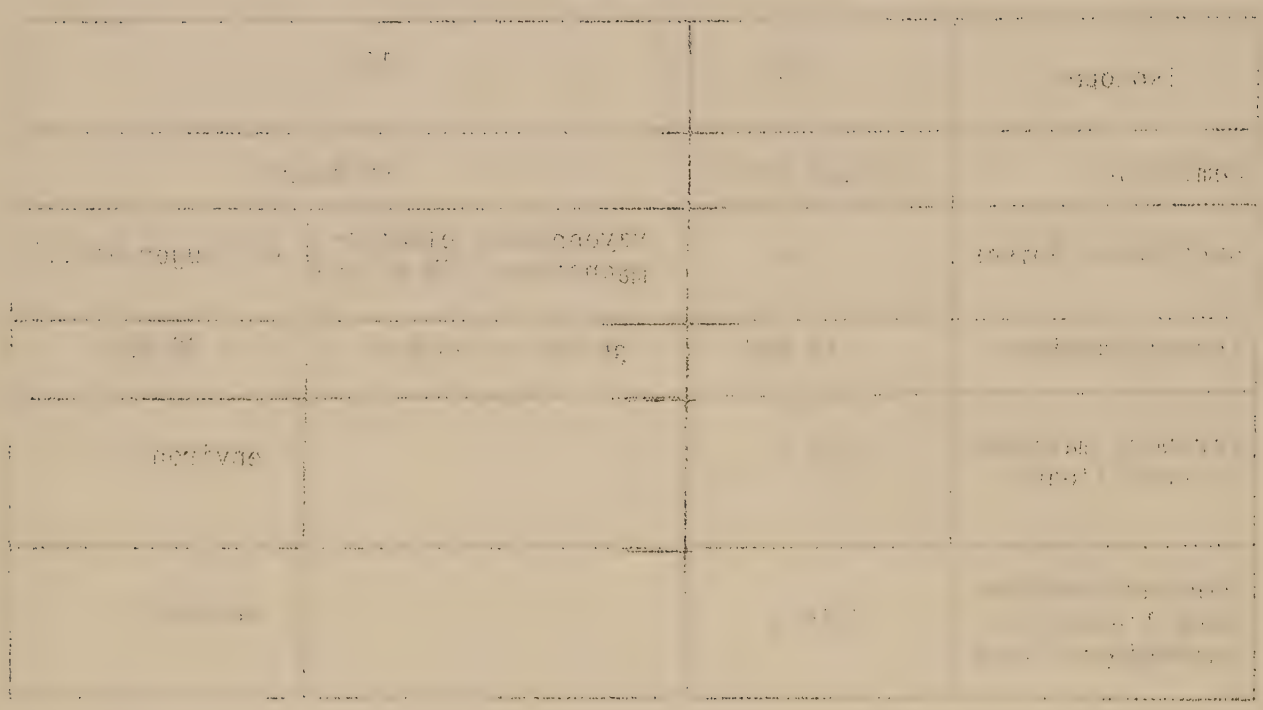
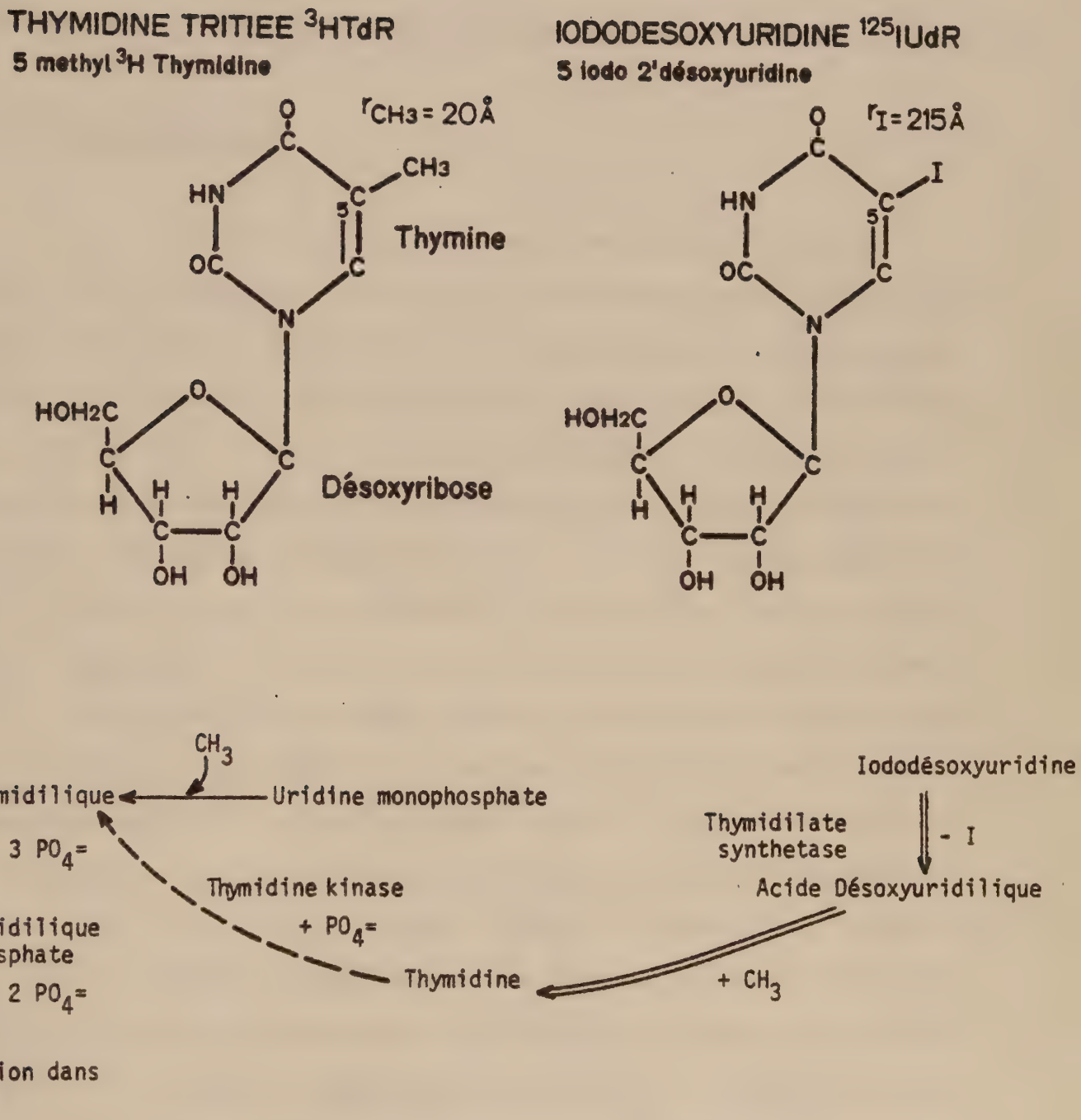


Figure 6 : STRUCTURE BIOCHIMIQUE ET MECANISMES D'INCORPORATION DE LA THYMIDINE TRITIEE ET DE L'IODODEOXYURIDINE.



grande proportion des électrons mis ayant un faible parcours (0,5 µ), les cellules ayant incorporé l'^{125}I peuvent donc être détectées par autoradiographie. L'émission de rayonnement γ permet également de détecter la présence de ^{125}I par comptage direct de la radioactivité dans un scintillateur solide (114,116).

4.4.2. L'autoradiographie

Une fine couche d'émulsion photographique (épaisseur moyenne 2 µ) est déposée à la surface de la lame portant une préparation cellulaire. Cette émulsion photographique est caractérisée par une forte concentration en sels d'argent (environ 80%) et une granulation très fine (diamètre des grains inférieur à 0,3 µm). L'émulsion est composée d'une phase cristalline contenant principalement du bromure d'argent, et de gélatine constituée de carbone, d'hydrogène, d'oxygène et d'azote. Du soufre est également présent dans la gélatine et, en tant qu'impureté, à la surface des cristaux de bromure d'argent (Figure 7). L'interaction des particules β émises par le tritium ou la ^{125}I, avec les cristaux de bromure d'argent, provoque une ionisation de ceux-ci. Les électrons ainsi libérés circulent dans les bandes de conductivité de l'émulsion, jusqu'à ce qu'ils soient captés dans les "points de sensibilité" du réseau cristallin, ces points de sensibilité correspondant à la présence d'impureté. Les électrons excédentaires réduisent les ions Ag^+ et l'atome d'Ag ainsi produit constitue l'image latente. Ce phénomène se déroule pendant l'exposition à +4°C des autoradiogrammes. Cette image est rendue visible par le processus de développement photographique, qui aboutit à une augmentation de la taille des grains d'argent métallique. La Figure 7 représente l'ensemble

de ces phénomènes. L'émulsion Ilford K2 a été utilisée dans nos premières expériences. Cependant elle est peu sensible et il était nécessaire d'exposer pendant 3 à 4 semaines les autoradiogrammes. C'est pourquoi nous avons modifié notre technique dans les expériences ultérieures, utilisant l'émulsion Ilford K5, plus sensible, et une technique de "développement par activation à l'or" (70) (Figure 7), ce qui nous a permis de ramener à 2 semaines la durée d'exposition des autoradiogrammes (106, 117, 118).

4.4.3. Détermination de la durée du cycle mitotique et de ses différentes phases par la courbe de l'évolution du pourcentage de mitoses marquées en fonction du temps (119).

C'est la méthode la plus utilisée pour mesurer la durée des différentes phases du cycle mitotique. Le principe de cette méthode est de marquer, par une injection unique de ^{3}HTdR, toutes les cellules de la population qui se trouvent en S, et de suivre le passage de ces cellules marquées à travers le compartiment des mitoses. On obtient une courbe en créneaux (Figure 8) , qui permet d'évaluer la durée des différentes phases. Cependant, une telle courbe est théorique, car son obtention exige que les conditions de marquage spécifiées précédemment page 41 soient respectées. D'autre part, on considère que les durées des différentes phases du cycle mitotique sont égales pour chaque cellule et que la durée de la mitose est négligeable.

En réalité, dans les systèmes biologiques, les durées des

phases du cycle d'une cellule à l'autre sont variables. On obtient donc expérimentalement des courbes sinusoïdales, dans lesquelles souvent seul le premier pic est bien défini. C'est pourquoi il a été nécessaire de recourir à des modèles mathématiques pour interpréter les courbes de mitoses marquées obtenues expérimentalement. Le principe général de ces modèles est de simuler une expérience de mitoses parquées à partir de valeurs obtenues expérimentalement et à partir de certaines hypothèses. On essaie ensuite d'ajuster au mieux la courbe théorique obtenue à la courbe expérimentale, en modifiant progressivement certaines valeurs théoriques.

Les modèles les plus couramment utilisés sont issus de la méthode de Barrett(120) qui est basée sur l'hypothèse que les durées pendant lesquelles les cellules sont en G_1, S et G_2 sont décrites par des distributions indépendantes log-normales. La méthode de Valleron *et al.* (121) qui est celle que nous avons utilisée, est dérivée de celle de Barrett. Elle incorpore, en plus, la possibilité de mort ou de perte cellulaire à n'importe quelle phase du cycle mitotique, ainsi que la production de cellules non proliférantes. Enfin, elle permet de déterminer la précision avec laquelle les paramètres sont mesurés.

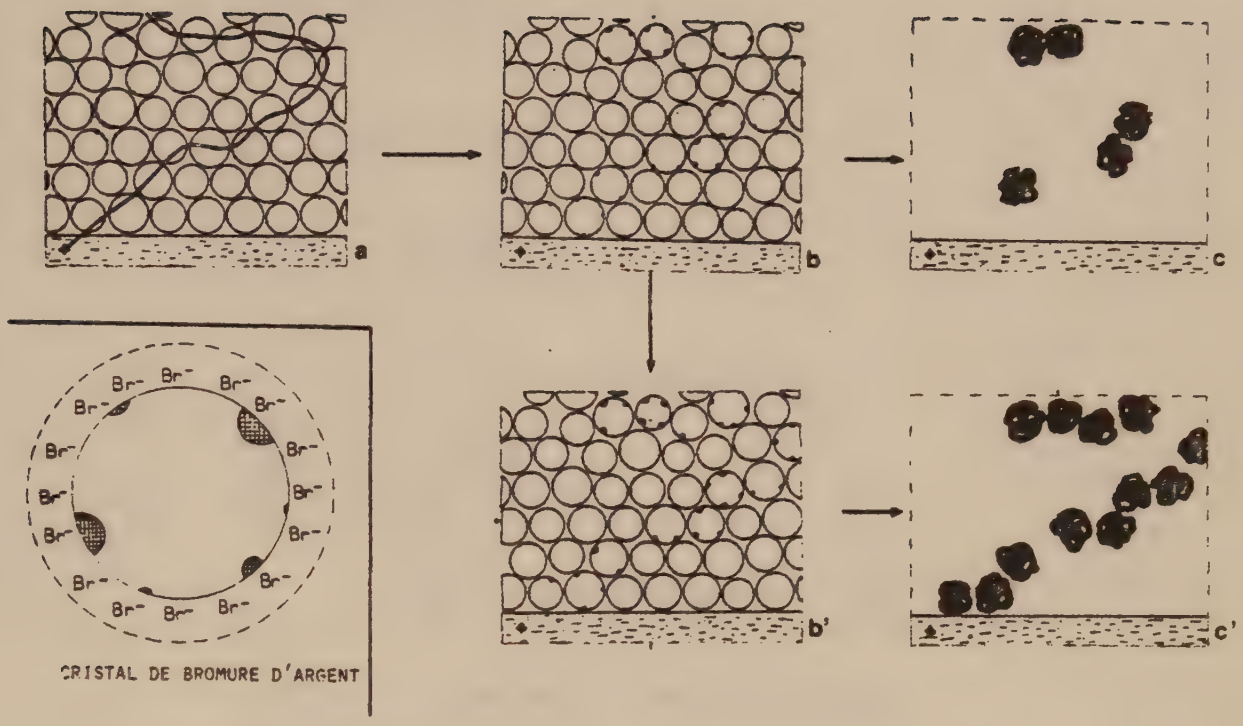


Figure 7 : Principe de la formation d'une image latente dans des émulsions autoradiographiques (d'après Rechenman)

La ligne continue pénétrant dans l'épaisseur de l'émulsion nucléaire (a) représente la trajectoire d'un électron émis par un marqueur radioactif. L'image latente formée par les microcristaux de Bromure d'Argent (b) résulte en une trace limitée et lacunaire (c) si elle est développée directement. Si elle est développée par activation, dans une solution contenant un sel d'or (b'), elle résulte en une trajectoire plus longue et plus continue (c')

▩ sites de sensibilité = imperfections dans le cristal d'AgBr (sulfures...), où se constitue l'image latente.

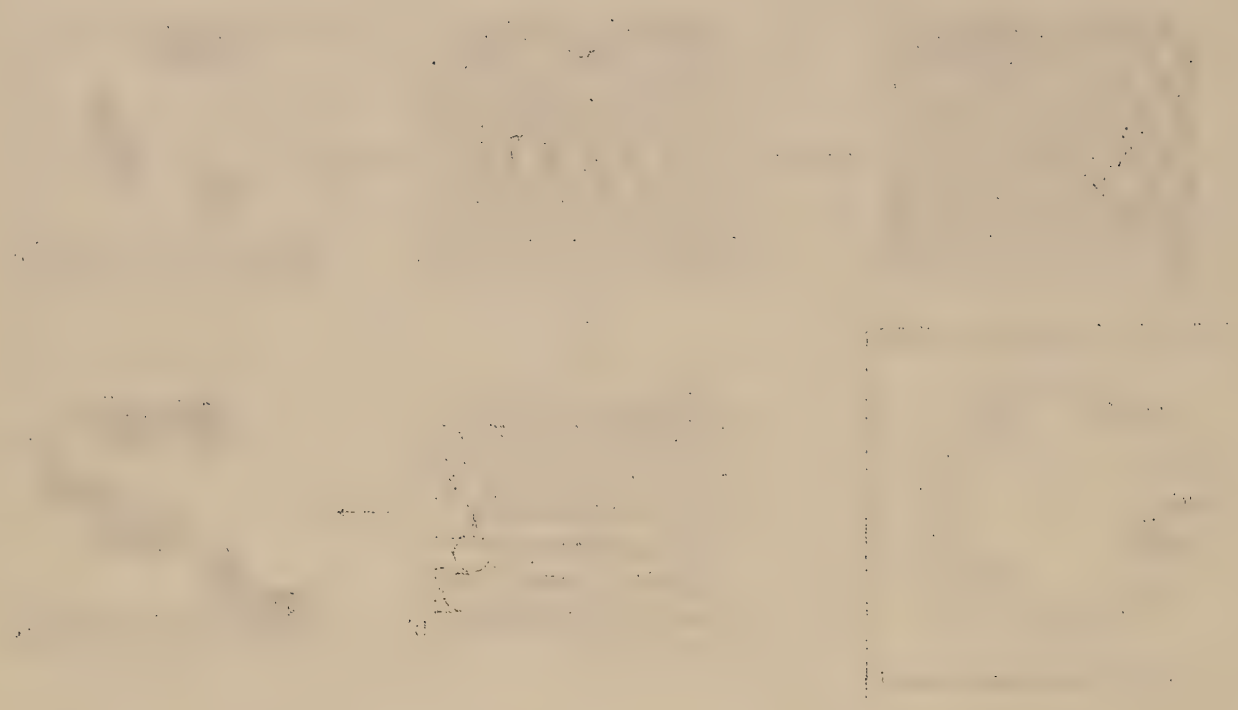
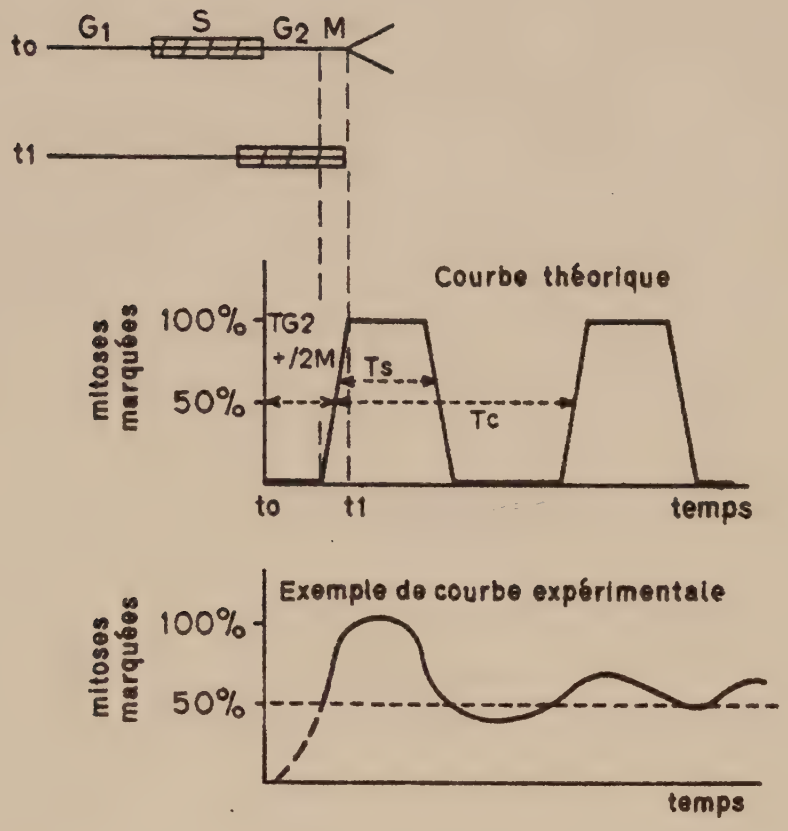


Figure 8 : DETERMINATION DE LA DUREE DU CYCLE MITOTIQUE PAR LA METHODE DES MITOSES MARQUEES



COURBE THEORIQUE

Au temps t_o : les cellules en S sont marquées. Aucune mitose n'est marquée.

A l'instant t_1 : la cohorte de cellules marquées a progressé dans le cycle : les cellules qui étaient en fin de S à l'instant t_o sont arrivées en mitose ; il apparait des mitoses marquées. Les premières cellules marquées arrivent en mitose après un temps égal à la durée de G_2.

COURBE EXPERIMENTALE

La durée des diverses phases varie d'une cellule à l'autre. La courbe s'amortit donc rapidement et tend vers une valeur constante, égale à Ts/Tc.

RESULTATS

Pour exposer les résultats que nous avons obtenus, nous incluons les principaux articles traitant du sujet de cette thèse, et qui ont été publiés dans des revues internationales ayant un comité de lecture. Nous faisons précéder chacun de ces articles d'un résumé en français.

1. Cinétique de la prolifération cellulaire dans la leucémie induite par le virus de Friend polycythémiant.

1.1. Study of the cellular proliferation of Friend leukemia

F. Smadja-Joffe, C. Jasmin, E.P. Malaise, C. Bournoutian. Int. J. Cancer, 11, 300-313, 1973.

RESUME : Etude cinétique de la prolifération cellulaire dans la leucémie de Friend.

Nous avons étudié les paramètres du type polyglobulique de la leucémie de Friend sur des souris DBA_2 inoculées avec le virus. La prolifération se produit surtout dans les érythroblastes de la rate. La durée moyenne de leur cycle mitotique est égale à 7,6 heures et le coefficient de prolifération cellulaire de la population splénique atteint 64%. Le taux de prolifération n'est pas affecté, même pendant les heures qui précèdent la mort. La multiplication des érythroblastes dans la rate résulte en trois phénomènes différents : 1/3 des érythroblastes produisent des érythrocytes qui vivent peu de temps ; une très faible fraction (5%) est responsable de la lente dilatation de la rate et de l'invasion progressive du foie et du sang ; les 2/3 disparaissent, ce qui est apparemment dû à une mort massive des cellules spléniques.

Int. J. Cancer: 11, 300-313 (1973)

STUDY OF THE CELLULAR PROLIFERATION KINETICS OF FRIEND LEUKEMIA

by

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We have studied the kinetic parameters of the polycythemic variety of Friend leukemia using DBA_2 mice inoculated with the virus. Proliferation was mainly observed in the erythroblasts of the spleen. The mean duration of the mitotic cycle is 7.6 h and the majority of erythroblasts are in active stages of the cell cycle. The proliferation rate is unaffected even during the hours preceding death. The multiplication of erythroblasts in the spleen results in three different phenomena: 1/3 of the erythroblasts produce short-lived erythrocytes; a very small fraction (1/20) of the proliferation accounts for the slow growth of the spleen and the progressive invasion of the liver and the blood; 2/3 disappear, and this loss is apparently due to massive splenic cellular death.

Leukemia induced by Friend virus in mice is characterized by a very prominent erythroblastosis localized principally in the spleen. It is in this organ that the first pathological cells appear. During the course of the disease, invasion of the spleen becomes total (Friend, 1957) provoking splenomegaly. Among the organs invaded secondarily by these leukemic cells are the liver, which can, however, become an erythropoietic organ *per se*, in splenectomized mice (Metcalf *et al.*, 1959), as well as the blood which is characterized by marked lymphocytosis (Metcalf *et al.*, 1959; Zajdela, 1962). During the terminal stage of the disease leukemic cells are disseminated throughout the organism, especially in the kidneys and lungs (Metcalf *et al.*, 1959).

Cytological (Zajdela, 1962) and physiological (Mirand *et al.*, 1968; Tambourin *et al.*, 1969) studies have already shown the capacity of leukemic cells for differentiation, and this has recently been confirmed by the measurement of the production of differentiated cells (reticulocytes, erythrocytes) in the peripheral blood (Tambourin *et al.*, 1973). In spite of their ability to differentiate, Friend erythroblastic cells are neoplastic cells, since erythroblastosis is only partly repressed by injection of washed red blood cells (Tambourin *et al.*, 1971). Following the isolation of the polycythemia-inducing Friend virus by Mirand *et al.* (1961) two strains of Friend virus are now known. In the two kinds of Friend leukemias, a stimulated erythroblastosis is observed, and red cell production in peripheral blood is abnormally increased. In the case of the anemic Friend leukemia, it has been shown that lowered hematocrit results from hypervolemia (Gallien-Lartigue *et al.*, 1970; Tambourin *et al.*, 1973).

However, a comprehensive study of proliferation and differentiation in Friend disease requires a knowledge of the characteristics of proliferation of leukemic cells. Using autoradiographic techniques it was thus the aim of our investigation to determine the parameters regarding the kinetics: (1) the proliferation of leukemic cells in the spleen, which appears to be the primary organ of their production, and in the liver and peripheral blood; (2) the growth characteristics of the leukemic spleen considered as a tumor.

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MATERIAL AND METHODS

The polycythemia-inducing strain of Friend virus has been routinely passaged in DBA_2 mice for 6 years in our laboratory. Viral suspension injected into the animals was obtained from homogenates of leukemic spleens ground with an Ultraturrax mixer in a solution of PBS and 0.5 M saccharose. The final supernatant, obtained after two centrifugations at $5,000 \times g$ for 10 min each, was preserved in liquid nitrogen until use. The titre of the viral preparations expressed in Spleen Dose 50% (SD_{50}) was determined using the technique of Rowe and Brodsky (1966). In our experiments, each mouse was inoculated IV with 0.2 ml of viral suspension containing 50 SD_{50}.

Female DBA_2 mice, approximately 2 months old and weighing 18-20 g, were used. The mean spleen weight of a normal mouse is 100 mg (106.5 ± 13.8). Tritiated thymidine was obtained from C.E.A. (Saclay, France). Its specific activity was from 7.6 Ci/mM to 9.3 Ci/mM. The radioactive solution was diluted to the appropriate concentration with 0.9% sodium chloride and 0.2 ml was injected into the orbital sinus of each mouse.

To study the mitotic cycle, we gave each leukemic mouse 2.5 Ci/g of tritiated thymidine. Half an hour after injection, and then every hour for 25 h, four leukemic animals were killed. In another experimental group, 1 μCi/g labelled thymidine was injected every 3 h for 15 h. Four leukemic mice were killed 1 h after each injection. With the doses of tritiated thymidine used, labelled spleen cells had a modal number of 30 to 35 grains per cell 15 days after exposure; this value was determined on 2,000 cells per point.

After the mice had been anesthetized with ether, organs under study were removed and cut transversely along the axis into several pieces with a scalpel, and imprints were made on four different slides for each organ. The imprints were fixed for 1 h in Carnoy's fixative (ethanol/acetic acid 3V/1V). This technique allowed the adhesion of a continuous monolayer of morphologically well-preserved cells on the glass slide (Fig. 1).

Labelling indices obtained on imprints, on histological sections, and on smears of spleen

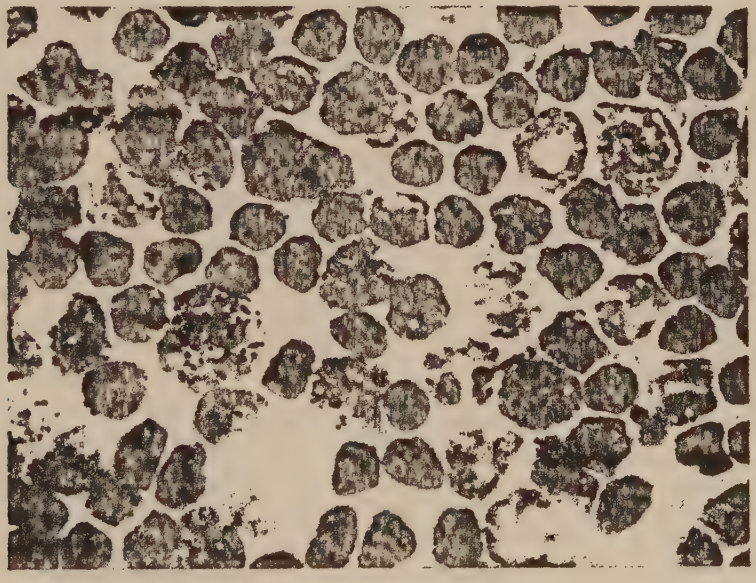

FIGURE 1

Autoradiograph of a Friend leukemic spleen, 15 days after the injection of 50 SD_{50} of viral suspension, showing a continuous monolayer of erythroblasts and proerythroblasts, with some granulocytic cells. (×640 Romanovsky staining).

and liver, were compared. They were determined by counting 2,000 cells per mouse. Mitotic indices and the percentage of labelled mitosis were calculated on the basis of 400 mitoses (100 per mouse) from imprints of spleen, the main organ where pathological proliferation is observed.

Peripheral blood was drawn into heparin from a section of the axillary artery of mice anesthetized with ether and blood smears were fixed for 10 min in absolute methanol. Labelling indices of peripheral blood cells were determined on 1,000 nucleated cells per mouse. Each point on the curves represents the mean of the results obtained on four mice.

Slides were dipped in a non-diluted Ilford K2 autoradiographic emulsion, heated once to 45° C, and exposed for approximately two weeks at +4° C. They were viewed for 5 min in a Kodak 19B viewer and fixed with Kodak acid fixative for 10 min. After dehydration with ethyl alcohol for a minimum of 1 h they were stained with Romanovsky stain.

Differential counts of cell populations were made also from imprints, smears, and histological sections of non-autoradiographed organs. The background of the emulsion was determined on autoradiographs of control imprint slides. Usually, under our experimental conditions, a cell was considered labelled when there were three grains over its nucleus.

Growth curves for the spleen, liver, and nucleated blood cells have been established. Of the mice inoculated with 50 SD_{50} of viral suspension given intravenously, seven DBA_2 mice were killed on days 4, 7, 21, and 28 after injection with the virus. Spleens and livers were weighed and counts of nucleated cells per mm^3 of peripheral blood were performed in a Malassez counting chamber.

Daily production rate of reticulocytes in peripheral blood was calculated with the formula:

$$\frac{\text{Total number of reticulocytes in peripheral blood}}{\text{Mean life-span of reticulocytes in peripheral blood}}$$

(Tambourin *et al.*, 1973).

The number of reticulocytes for 1,000 erythrocytes was determined with water-soluble brilliant Cresyl blue, dissolved in fetal calf serum. Erythrocyte counts in a Malassez counting chamber were duplicated. The blood volume was determined by the ^{59}Fe labelled red cell dilution method (Paxson and Smith, 1968; Tambourin *et al.*, 1973). The mean life-span was determined according to Ganzoni *et al.* (1969) and Tambourin *et al.* (1973). The donor red cells, from leukemic mice, were washed three times and adjusted to a hematocrit of 70%. They were injected intravenously into uninfected polycythemic recipients with hematocrits between 70 and 75%.

Duration of the mitotic cycle and its phases has been determined following the method of Quastler and Sherman (1959) with the aid of a mathematical model described by A. J. Valleron (Valleron, 1973). In this model, all phases of the cell cycle may vary; the distribution of individual values is log normal and distributions of values from different phases are considered as independent. Numerous values of means and standard errors from phases of the mitotic cycle, G_1, S and G_2 are generated. The most probable theoretical curve is the closest curve fitting with experimental values.

RESULTS

Evolution of spleen and liver weights and of the number of nucleated cells in the peripheral blood in leukemic mice

Growth curve and cytological formula of the spleen. Growth of the leukemic spleen is composed of two distinct phases (Fig. 3). The exponential phase lasts from the 7th to the 21st day after injection of 50 SD_{50} of viral suspension, characterized by a doubling time of 6 days. Beginning with the 21st day the slope of the growth curve diminishes, and this second phase is called the terminal phase, characterized by a doubling time of 18 days. The mice begin to die 30 days after injection of virus, the mean weight of the spleens then being 2.5 ± 0.6 g.

Cytological formulae determined from imprints of leukemic spleens taken during the phase of exponential growth (spleen weighing approximately 1 g) and during the terminal phase of the disease (spleen weighing more than 2 g), as well as those of normal spleens, are reported in Table I.

Leukemic spleens are essentially erythroblastic with 70 to 90% of the cells belonging to this line (" erythroblastic cells " include: hyperbasophilic

KINETIC STUDY OF FRIEND LEUKEMIA

TABLE I

CYTOLOGIC FORMULAE AND MITOTIC INDICES OF NORMAL AND LEUKEMIC SPLEENS FROM IMPRINTS AND SMEARS [1]

		Normal mice	Leukemic mice		
Days after virus injection		0	14	28	
Mean spleen weights (mg)		106±13.8	860±49	2090±181	2090±181
Technique used		Imprint	Imprint	Imprint	Smear
% of erythroid cells	Proerythroblastic	2.52	48.46	51.94	45.73
	Erythroblastic	15.00	35.14	32.92	34.99
	Total number	17.50±5.30	83.60±5.74	84.86±3.20	80.79±6.62
% of reticular cells		1.50±0.71	2.64±1.96	1.79±0.62	10.29±5.72
% of lymphoid cells		77.00±5.76	14.50±3.04	12.79±3.26	8.93±0.76
% of granulocytic and monocytic cells		2.88±0.63	<1	<1	<1
Mitotic indices			2.64±0.42	2.40±0.20	2.10±0.50

[1] Each value is the mean calculated on seven DBA_2 mice.

TABLE II

NUMBER AND TYPE OF NUCLEATED CELLS IN PERIPHERAL BLOOD OF NORMAL AND LEUKEMIC MICE

		Normal mice [1]	Leukemic mice [2]	
Days after virus injection		0	14	28
Mean spleen weights (mg)		106±14	860±49	2,060±181
Mean number of nucleated cells in peripheral blood		3,140±870	28,187±12520	140,000±23,000
% of polynuclear cells		14±8	11−33−35−44−50−	4−12.5−15−18−24−35−
% of lymphocytes		79±21	17−20−37−57−67−	13−13−28−29−33−40−
% of monocytic cells		7.8±4	2.6±3.2	1.7±2.3
% of erythroid cells	Proerythroblastic	0	1.76±1	7±1.7
	Erythroblastic	0	1−5−18.7−22−23−	16.6−24−45−59−67−72−
	Total number	0	2−6−18.7−25.8−26−	24−33−52−64.3−65.5−77
% of myelocytic cells		<1	7.4±4.3	2.0±2.8

[1] Normal mice: each value is the mean calculated on five mice.
[2] Leukemic mice: when the values are too scattered to have a meaning, individual data are given.

proerythroblasts (Zajdela, 1962), proerythroblasts, basophilic polychromatophilic and acidophilic erythroblasts). Although relatively diminished in comparison with the normal spleen, lymphoid line is augmented in absolute value. Finally, less than 5% of reticular, granular, and monocytic cells are found in either normal or leukemic spleen.

The same Table shows the cytological formula obtained with smears of 2.6 g spleens. One may note that the only clear difference in comparison with the results of the imprint (Table II) is an increase in the percentage of reticular cells at the expense of the lymphoid line.

Growth of the liver. The liver increases in weight later and more slowly than the spleen. It is 1 ± 0.1 g in normal mice and reaches 2 ± 0.2 g just before death in leukemic mice.

On microscopic examination the appearance of small islands of leukemic cells in perivascular zones can be observed beginning 1 week after viral infection (Fig. 2). These blast cells, which are very easily distinguished from hepatocytes, can almost entirely infiltrate the liver at a later stage of disease.

Nucleated cells in peripheral blood. The number of nucleated cells per mm^3 in peripheral blood increases regularly up to the 28th day after viral infection in contrast to the spleen weight which increases more slowly after the 21st day (Fig. 3).

As indicated on Table II, the peripheral blood is already invaded by erythroblastic cells when the spleen weighs about 1 g. Moreover, the lymphocytic population is greatly increased. It is at the advanced stage of leukemia, when there are more than 10^5 nucleated cells per mm^3 in the peripheral blood, that the erythroblastic compartment becomes very large, representing 24 to 77% of nucleated cells.

Daily output of reticulocytes in peripheral blood. As indicated in Table III, the daily production rate of reticulocytes in peripheral blood is 0.8×10^9 at the 14th day after viral infection, and it reaches 3×10^9 at the terminal phase of the disease.

FIGURE 2

Autoradiograph of a histological section of Friend leukemic liver, 1 week after the injection of 50 SD_{50} of viral suspension. We observe here an island of labelled leukemic cells, easily recognizable among unlabelled hepatocytes. ($\times 640$ Hematoxylin—eosin staining).

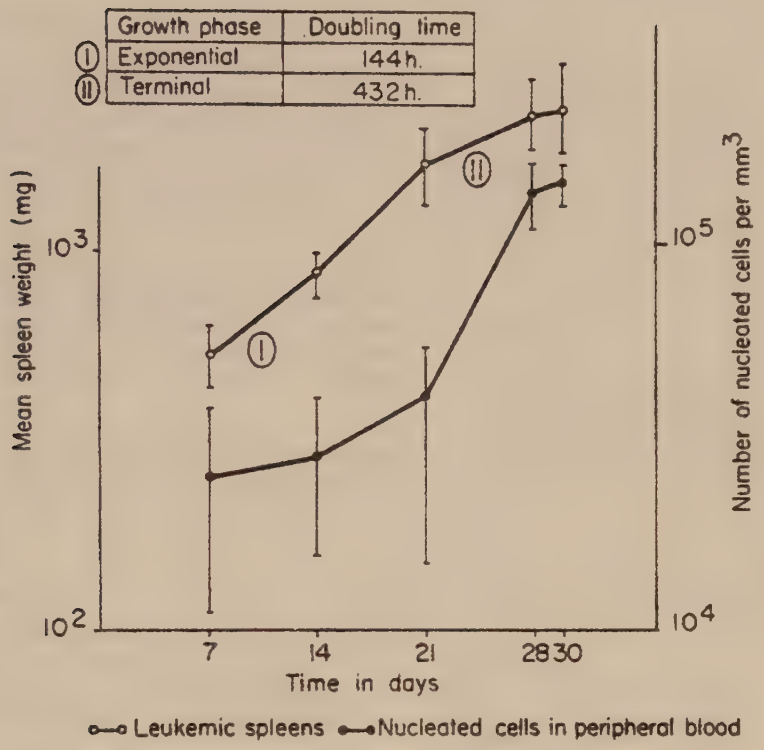

FIGURE 3

Evolution of the mean spleen weights and of the number of nucleated cells in the peripheral blood after Friend virus injection (50 SD_{50} on day 0). Each point represents the mean calculated from 10 mice.

TABLE III

DAILY OUTPUT OF RETICULOCYTES IN PERIPHERAL BLOOD [1]

	Normal mice	Leukemic mice	
Days after virus injection	0	14	30
Mean spleen weight (mg)	106±14	860±49	2,500±100
Hematocrit (%)	47±1	41.7±3	57±3
Number of erythrocytes (10^6/ml)	8.7±0.4	8.6±0.8	9.7±0.8
Blood volume (ml)	1.44±0.13	1.5±0.2	2.1±0.4
Total number of erythrocytes (10^9)	12.5	12.9	19.9
% of reticulocytes	3.8±0.5	14.1±4	29±6
A = total number of reticulocytes ($\times 10^8$)	0.4	1.2	5.8
B = Mean life-span of reticulocytes in peripheral blood	30	36	36
Daily production rate of reticulocytes in peripheral blood $= \frac{A}{B}$ ($\times 10^8$)	0.33	0.81	3.1

[1] Each value is the mean calculated on 10 mice.

Study of the phase of exponential growth

Duration of the mean mitotic cycle of spleen cells. The curve of labelled mitoses (Fig. 4A) presents two well-defined peaks. The maximum of the first peak is observed at 4 h; it reaches 90%. The second appears at 10 h reaching 80%. The ascending phase of a third peak begins to be visible at 17 h.

The minimal values obtained are never inferior to 50%. Therefore we have determined the durations of the various phases of mitotic cycle with the aid of a mathematical model described by Valleron *et al.* (1973). The results are as follows:

Duration of mitotic cycle (which corresponds to the mean mitotic cycle of cell populations of leukemic spleen):

$T_c = 7.6$ h.

Durations of

DNA synthesis phase: $T_s = 5.40 \pm 1.14$ h.
Post-synthetic phase: $T_{G2} = 1.10 \pm 1.29$ h.
Mitotic phase: $T_M = 0.5 \pm 0.00$ h.
Pre-synthetic phase: $T_{G1} = 0.6 \pm 1.09$ h.

Growth fraction GF has been estimated by the formula (Mendelsohn, 1962):

$$GF = \frac{LI}{T_s/T_c}$$ where LI is the labelling index calculated on spleen imprints 1 h after tritiated thymidine injection

$$GF = \frac{0.45}{5.4/7.6} = 0.64.$$

The potential doubling time ($T_{D.pot}$) of the leukemic spleen is calculated by the formula (Steel, 1968):

$$T_{D.pot} = \lambda \cdot \frac{T_s}{LI} = 0.7 \cdot \frac{5.4}{0.45} = 8.4 \text{ h.}$$

The experimental doubling time (T_D) of the leukemic spleen being then 144 h, the cell loss (Steel, 1968) may be estimated as:

$$1 - \frac{T_{D.pot}}{T_D} = 1 - \frac{8.4}{144} = 0.94.$$

Labelling index of the spleen, leukemic cells of the liver, and nucleated cells of the peripheral blood. As reported in Table IV, labelling indices,

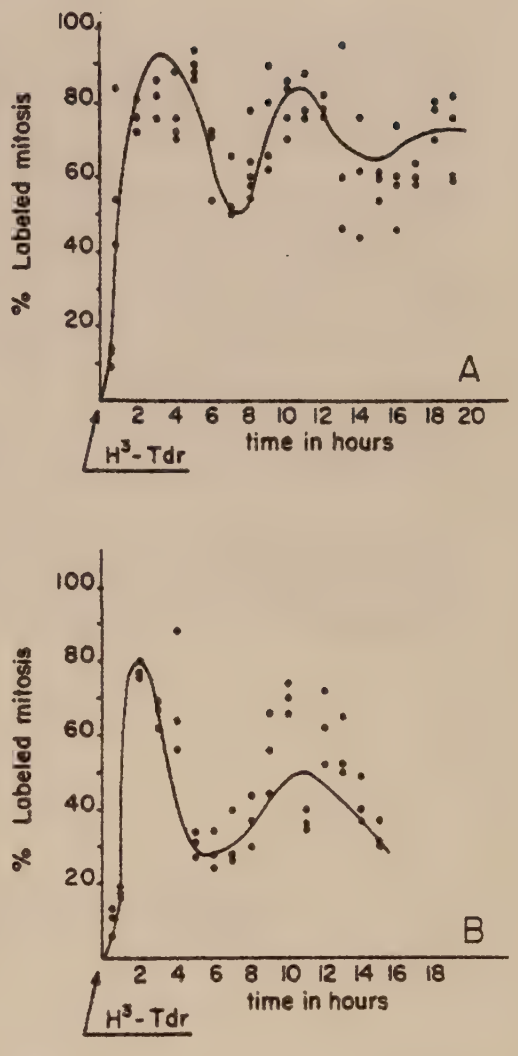

FIGURE 4

Curves of labelled mitosis of leukemic spleen cells, optimalized with a mathematical model. 2A: during the exponential growth of the disease. 2B: at the terminal stage of the disease. The tritiated thymidine (2.5 μCi/g) is injected at time 0. Counts are done on 400 mitoses for each point.

after a single injection of tritiated thymidine, are not significantly different when calculated on spleen imprints, smears or histological sections. Further studies, therefore, were based on imprint examination. One half hour after injection of the radioactive marker, 50% of spleen cells and

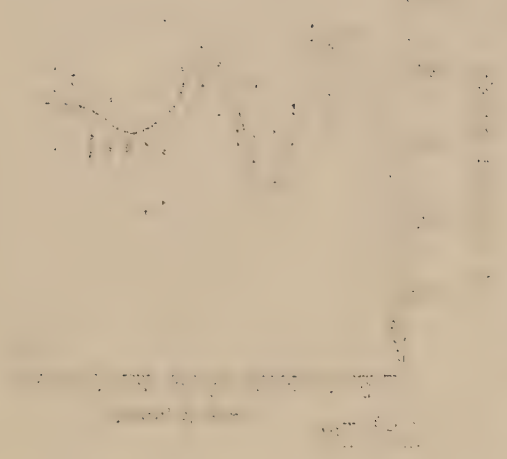

TABLE IV

LABELLING INDICES DETERMINED ON IMPRINTS, HISTOLOGICAL SECTIONS AND SMEARS OF LEUKEMIC SPLEENS

Mean spleen weight (mg)	Imprints	Histological sections	Smears
1,300±125	42.2±3.1	38.0±3.0	40.5±2.9

[1] Each value is the mean calculated on four DBA_2 mice, 20 days after injection of 50 SD_{50} of Friend virus. The labelling index is determined on 2,000 cells for each mouse.

60% of leukemic cells of the liver are labelled. After an hour the percentage of labelled leukemic cells is highest in the liver (75%). In this organ it decreases only after 15 h, reaching about 50% at the end of the observation period. The labelling index of leukemic spleen cells reaches the same maximum value (70%), although it does so more slowly; it does not decrease during the 25 h following injection (Fig. 5). The modal number of grains per cell, 32.5 half an hour after injection of tritiated thymidine, is 15.0 at the 18th hour and 12 at the 48th hour (Fig. 6).

When several injections of tritiated thymidine are given, 90% of leukemic spleen cells are labelled within 7 h of the first injection. No more than 25% of labelled spleen cells are observed in normal spleens. During a period of 15 h of labelling there are no further changes in the percentage of labelled cells after the 7th hour.

In the liver, where only leukemic cells are observed, the percentage of labelled cells is 93% at the 7th hour and increases up to the 16th hour, when it reaches a maximum of 97% (Fig. 7).

In contrast, only a small proportion of leukemic cells from peripheral blood are labelled. In fact, as shown in Figure 8, less than 7% of all nucleated cells and 1% of erythroblastic cells are

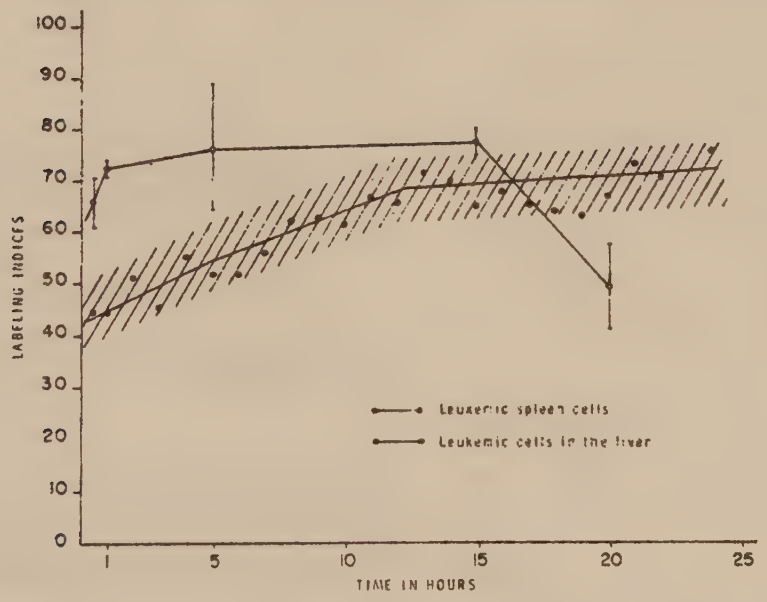

FIGURE 5

Labelling indices of leukemic spleen cells and of the leukemic cells of the liver, after a pulse of 50 μCi/g of tritiated thymidine.
Four mice were killed each hour. Labelling indices were determined on 2,000 cells for each mouse.

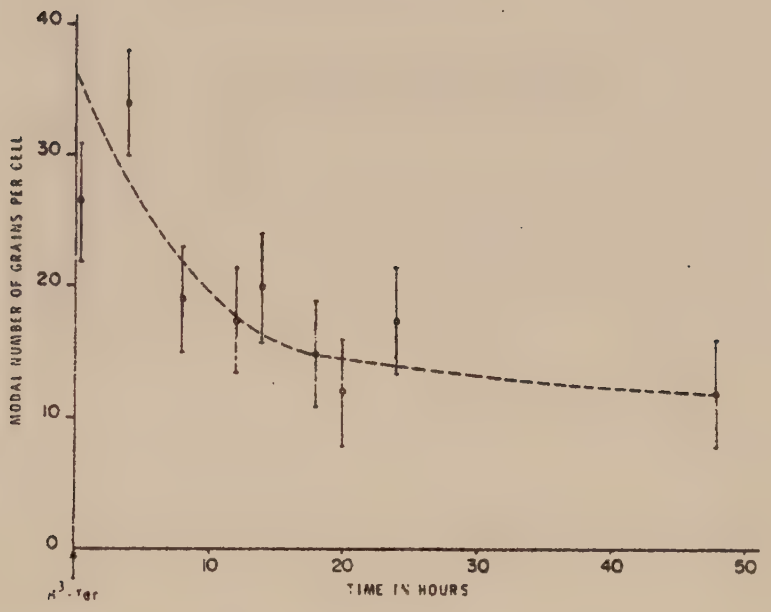

FIGURE 6

Evolution of the modal number of grains per cell in the leukemic spleens after a pulse of tritiated thymidine. Each number was determined on 200 labelled cells.

labelled 1 hour after tritiated thymidine injection. This labelling index increases slightly, reaching at 50 h a maximum of 15% for all nucleated cells and 25% for erythroblastic cells.

Study of the terminal phase of the disease

Duration of the mean mitotic cycle of spleen cells. One half hour after a single injection of tritiated thymidine the percentage of labelled spleen cells is 30%.

The curve of labelled mitoses presents a first peak at 3½ h with a maximum value of 75% and a second peak whose maximum is 50% at 11½ h (Fig. 4B). The following parameters have been calculated as described earlier and are:

Duration of the mean mitotic cycle and of its various phases:

$T_c = 7.5$ h

$T_s = 2.91 \pm 2.19$ h

$T_{G2} = 1.36 \pm 0.54$ h

$T_M = 0.25 \pm 0.00$ h

$T_{G1} = 2.98 \pm 2.18$ h

Growth fraction:

$$GF = \frac{LI}{T_s/T_c} = \frac{0.30}{2.9/7.5} = 0.78.$$

Potential doubling time:

$$T_{D,pot} = \lambda \frac{T_s}{LI} = 0.7 \cdot \frac{2.9}{0.3} = 6.76.$$

Cell loss:

The experimental doubling time (T_D) being then 432 h, the cell loss is estimated as:

$$1 - \frac{T_{D,pot}}{T_D} = 1 - \frac{6.76}{432} = 0.98.$$

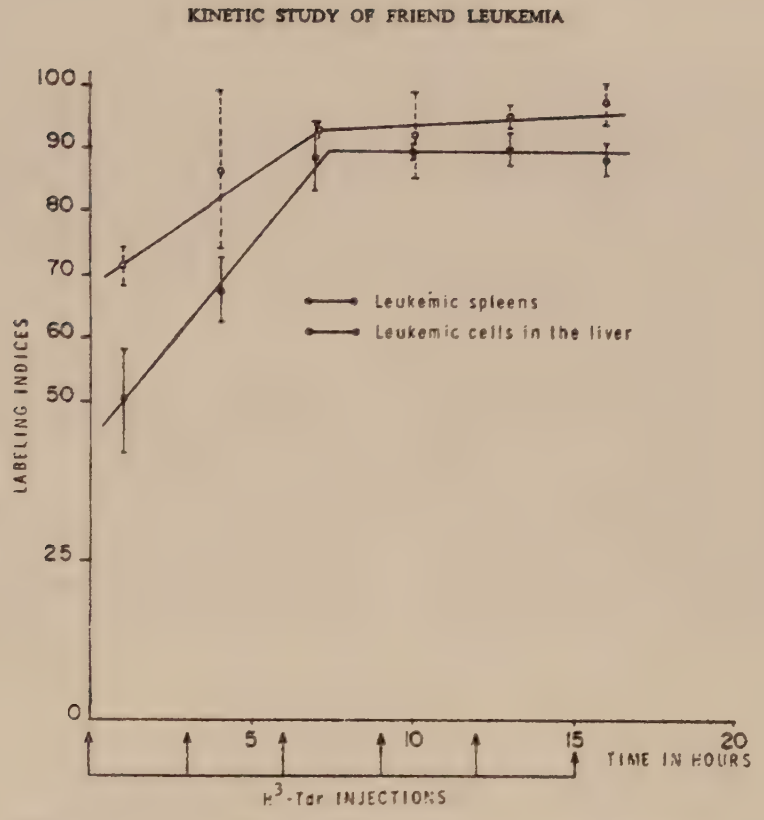

FIGURE 7

Labelling indices of leukemic spleen cells and of the leukemic cells of the liver, during the exponential stage of the disease. 1 μCi of tritiated thymidine was injected every 3 h for 15 h. One hour after each injection, four mice were killed. Each index was determined on 2,000 cells.

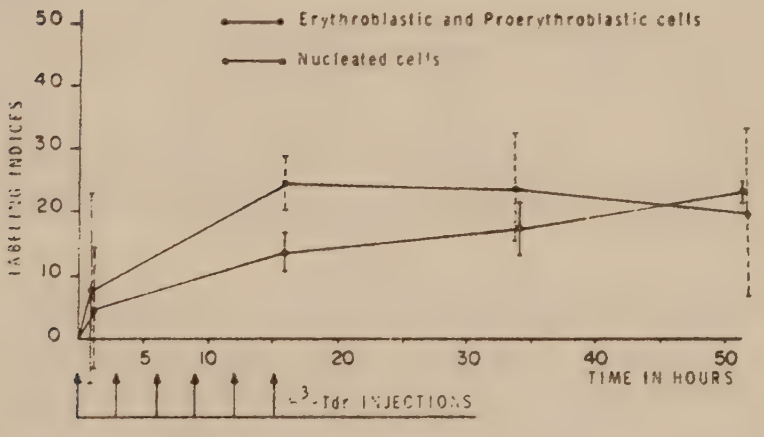

FIGURE 8

Labelling indices of nucleated cells and of erythroblastic cells (proerythroblastic and erythroblastic) in the peripheral blood during the exponential stage of the disease. 1 μCi/g of tritiated thymidine was injected every 3 h for 15 h. Four mice were killed 1 h after each injection and at 34 h and 52 h after the first one. Each point was determined on 1,000 cells.

DISCUSSION

The duration of the mitotic cell cycle has been studied in a heterogenous population of splenic cells. Nevertheless, since a spleen invaded by Friend leukemia contains a minimum of 80% of erythroblastic cells, it seems very probable that our results using the Quastler method (Quastler *et al.*, 1959) are valid for Friend leukemic cells.

One fundamental aspect of the kinetics of proliferation in Friend leukemia is the existence of an intense multiplication of erythroblastic cells in the spleen. This cellular proliferation is characterized by a very short cell cycle and by a growth fraction which is nearly 100% (Table V). The duration of the cycle does not seem to be characteristic of this disease; indeed cycles of comparable duration have been described in bone-marrow cells from normal mice (Frindel *et al.*, 1967) or other mammals (Odell, 1968; Bond *et al.*, 1962) as well as for cells from spontaneous lymphoma in AKR mice or L 1210 leukemia (Metcalf and Wiadrowski, 1966; Choquet *et al.*, 1970) (Table VI). The duration of the mitotic cycle is not apparently modified during Friend leukemia. At the terminal stage of the disease, we only observe a more important variation of the mitotic cycle phase durations. Moreover, if the existence of a large growth fraction is not surprising 15 days after the injection of the virus (Lala and Patt, 1966), it is remarkable to note that this intense proliferation does not slow down even on the eve of death when the spleen weight is no longer increasing. It has been in fact noticed in the majority of neoplastic processes that a late slowing down of cellular proliferation is associated either with a lengthening of the cell cycle in the case of ascites (Frindel and Tubiana, 1967) and transplanted leukemias (Choquet *et al.*, 1970), or with a reduction of the growth fraction in solid tumors (Frindel *et al.*, 1968). It is, of course, impossible to be sure whether this phenomenon has a general significance (as would be the case if it were associated with the viral character of Friend leukemia) or whether it is simply a characteristic of Friend leukemia.

While the spleen is the principal organ concerned with the proliferation of Friend cells, it seems that the liver is only invaded at a later stage. Furthermore, relatively few erythroblastic cells are circulating in the blood where, in general, they appear unable to multiply. One hour after the injection of tritiated thymidine the labelling index of peripheral blood was very low and remained so during repeated injections over an interval of 16 h and for an observation

TABLE V

PARAMETERS OF THE CELL PROLIFERATION IN FRIEND VIRUS-INDUCED LEUKEMIA DURING THE EXPONENTIAL GROWTH AND AT THE TERMINAL STAGE OF THE DISEASE [1]

Days after virus injection	Mean spleen weight (mg)	Duration of the mitotic cycle T_C (h)	TS	TG_2	TM	TG_1	% of labelled cells 1 h after ^{3}Htdr injection	Growth fraction (%)	Doubling time of the leukemic spleen (h)		Cell loss (%)
									Potential	Experimental	
14	860±49 (exponential phase)	7.6	5.4 (1.14)	1.10 (1.29)	0.5 (0.00)	0.60 (1.09)	45 (1.5)	64	8.40	144	94.2
28	2,090±181 (Terminal phase)	7.5	2.91 (2.19)	1.36 (0.54)	0.25 (0.00)	2.98 (2.18)	30 (1.4)	77.6	6.76	432	97.9

[1] Each value is the mean calculated on four DBA_2 mice. Duration of the various phases of the mitotic cycle: the mean values and the standard deviations (between parenthesis) were determined with the aid of a computer.

TABLE VI

SUMMARY OF KINETIC DATA OBTAINED ON DIFFERENT TUMORS

				Stage of the disease	T_c (hours)	GF (%)	Cellular loss (%)	Reference
Normal hematopoietic cells			Mouse bone-marrow blasts		8.5	86	—	6
			Rat megakaryocytes		9.3	—	—	18
			Dog erythroblastic cells		10.0	—	—	1
Murine leukemias	Transplanted		L 1210	Exponential	9.0	100	5	2
				plateau	14.0	88		
	Virus-induced		The spontaneous AK lymphoma	Advanced	8.0[1]			1
			Friend-virus-induced leukemia	Exponential	7.6	64	94.0	This
				plateau	7.5	75	98.0	paper
Burkitt lymphoma					—	90	70	9
						100		
Non-hematopoietic tumors	Ascitic		Erlich	1 day	—	82	—	
			NCTC (Ascites)	7 days	—	53	—	7
	Solid	Experimental	NCTC fibrosarcoma	Exponential	16.5	44	—	7
				Terminal	17.5	29	—	
		Human	Basocellular cancer of the skin		72	30	95	4
			Spinocellular cancer of the skin		38	40	90	4

[1] large lymphoma cell population.

period of 52 h. This suggests that erythroblastic cells in the liver and the peripheral blood only constitute an extremely small fraction of the cells produced in the spleen.

A comparison of the measured growth rate of the spleen with the rate of cell proliferation calculated from a formula currently used in the study of the kinetics of solid tumors (Steel, 1968) has shown that 95% of the cells produced in the spleen must in fact leave this organ. We have now shown that this cell loss cannot be explained by the presence of erythroblasts in the liver and peripheral blood. As an alternative hypothesis, the cellular loss could be considered to be the result of a differentiation of the leukemic erythroblastic cells, leading to short-lived erythrocytes. We have shown that, by the 14th day, the number of reticulocytes present in the peripheral blood is of the order of 10^9 (Table IB). From these data calculations show that 1 g of spleen is equivalent to 10^9 cells and produces an average of 3×10^9 cells per day. It follows that 2/3 of these cells disappear. Thus, 2/3 of the cells produced in the region of the erythroblasts in the spleen would appear to be lost. Similar cell losses have been recognized for a long time in solid tumors, whether experimental (Frindel *et al.*, 1968) or human (Frindel *et al.*, 1968; Iversen *et al.*, 1972). It is unfortunately not always possible to make a distinction between loss due to cellular differentiation and true cell death. From this point of view, however, the Friend leukemia model seems to be privileged.

Accompanying the large-scale cell lysis we have noticed a particularly slow decrease of the mean number of grains in labelled cells following a single injection of tritiated thymidine. The most satisfying explanation of this phenomenon is to postulate the existence of an exceptionally high reutilization of ^{3}HTdr related to a massive cellular lysis. Though the reutilization of ^{3}HTdr is a well-known phenomenon (Heiniger *et al.*, 1971), such high levels are not usually found.

ACKNOWLEDGEMENTS

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ÉTUDE CINÉTIQUE DE LA PROLIFÉRATION CELLULAIRE DANS LA LEUCÉMIE DE FRIEND

Nous avons étudié les paramètres cinétiques du type polycythémique de la leucémie de Friend sur des souris DBA_2 inoculées avec le virus. La prolifération se produit surtout dans les érythroblastes de la rate. La durée moyenne du cycle mitotique est de 7.6 heures et la majorité des érythroblastes en sont aux phases actives du cycle cellulaire. Le taux de prolifération n'est pas affecté, même pendant les heures qui précèdent la mort. La multiplication des érythroblastes dans la rate entraine trois phénomènes différents: 1/3 des érythroblastes produisent des érythrocytes qui vivent peu de temps; une très faible fraction (5%) est responsable de la lente dilatation de la rate et de l'invasion progressive du foie et du sang; les 2/3 disparaissent, ce qui est apparemment dû à une perte massive de cellules spléniques.

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1.2. Study of cell death in Friend leukemia

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Cell Tiss. Kinet., 9, 131-145, 1976.

RESUME : Etude de la mort cellulaire dans la leucémie de Friend.

La mort cellulaire dans la rate leucémique de Friend a été étudiée *in vivo* ; les cellules proliférantes ont été marquées par injection unique de ^{125}IUdR et de ^{3}HTdR. L'évolution en fonction du temps de la radioactivité spécifique de la rate a ensuite été déterminée par autoradiographie et en mesurant l'activité spécifique de l'ADN cellulaire. Ces deux techniques indiquent l'existence d'une grande réutilisation de l'^{3}HTdR (> 50%), détectable dès 7 heures après injection. Le taux de renouvellement de l'ADN des cellules de la rate leucémique est élevé (84% par jour). Ces résultats confirment que la majorité des cellules produites dans la rate leucémique de Friend sont rapidement perdues ; ils démontrent aussi que cette perte cellulaire est principalement due à une mort massive des cellules leucémiques , après une ou deux divisions. La majorité des cellules leucémiques de Friend sont donc caractérisées par une capacité de prolifération limitée et une courte durée de vie.

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STUDY OF CELL DEATH IN FRIEND LEUKAEMIA

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ABSTRACT

Cell death of splenic Friend leukaemic cells has been studied *in vivo*, using ^{125}I-UdR and ^{3}H-TdR pulse labelling. The evolution of the splenic specific activity has been measured by autoradiography and external counting during 40 hr after injection of the labelled precursor. These two techniques show the existence of a large reutilization of ^{3}H-TdR (50%), which is measurable as soon as 7 hr after the injection. The DNA turnover rate is rapid, 83·8% of the splenic cellular DNA being renewed per day. These results confirm that most of the cells produced in the Friend leukaemic spleen are rapidly lost; they also demonstrate that this cell loss is mainly due to a massive death, which occurs in proerythroblastic and erythroblastic compartments after one or two cell divisions.

Therefore, Friend leukaemic cells, are characterized by a limited capacity of proliferation and a short lifespan.

Cell death is a normal regulatory factor of the proliferation of healthy tissues. In general, cell death is equal to cell production and the total number of cells that comprises a tissue remains constant. In cancer, particularly in solid tumours, it has been shown that the rise in the number of tumour cells is accompanied by a cell loss. This is in part due to the migration of tumour cells and in part to their death. This loss is quite small during the exponential growth phase, usually less than 30% (Frindel *et al.*, 1967; Steel, 1968; Denekamp, 1970; Dethlefsen, 1971). Cell death is especially noticeable in the plateau phase of the growth of the tumour; it appears to be, at least in part, the consequence of the cells growing in an unfavourable environment, partly brought on by the size of the tumour, which becomes anoxic and deprived of nutrients (Tannock, 1968). A similar situation is observed when cells such as the L 1210 leukaemia are grown in an ascitic form. In this experimental model, which represents one extreme situation, the cell death is virtually nil during the exponential phase of the ascites and only intervenes in the plateau phase (Frindel *et al.*, 1969; Denekamp, 1970; Hofer & Hofer, 1971).

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Only a few studies have been made on the growth pattern of leukaemias and tumours induced by viruses. Up to the present, two autoradiographic studies have been made, one on a spontaneous lymphoma of AKR mice (Metcalf & Wiadrowski, 1966), the other on a leukaemia induced by Friend virus in mice (Smadja-Joffe *et al.*, 1973). They have shown that haemopoietic cells transformed by the virus multiply at the same speed as normal cells; in the case of Friend leukaemia the calculation based on the comparison of the real and potential doubling times of the leukaemic spleen has indicated that there is a cell loss factor of 94% during the exponential growth phase (Steel, 1968). Furthermore, a comparison of the level of production of leukaemic cells (proerythroblastic and erythroblastic) in the spleen, of their migration to other organs and of their rate of differentiation into erythrocytes has shown that at least two-thirds of the cells produced in the spleen die rapidly (Smadja-Joffe *et al.*, 1973).

We have studied this cell loss with a more rapid system for its quantification and we have obtained experimental evidence for the cell death. Such a study was all the more worthwhile since experimental data generally supported the view that these cells had a short viability *in vivo*. In particular, different investigators have shown that the cells are incapable of multiplying in non-irradiated isogeneic hosts (Friend, 1957; Metcalf, Furth & Buffett, 1959; Takada, Takada & Ambries, 1971) and it is only exceptionally that transplantable subcutaneous tumours can be obtained by injecting fragments of spleen from very leukaemic animals (Buffett & Furth, 1959; Friend & Haddad, 1960; Wendling, Tambourin & Bucan-Varlet, 1974). Our study has been based on the utilization *in vivo* of 5-iododeoxyuridine labelled with ^{125}iodine (^{125}I-UdR). It has been demonstrated that this molecule is specifically incorporated into the DNA of cells when they are in the S phase of the cell cycle (Hampton, Rich & Eidinoff, 1960; Prusoff, 1960), but only in small amounts (Hughes *et al.* 1964; Fox & Prusoff, 1965). Therefore, contrary to ^{3}H-TdR, ^{125}I-UdR is either very little or not reutilized after cell death (Hughes *et al.*, 1964).

We have measured the direct rate of loss of labelled cellular DNA from the spleen, and compared the time evolution of the splenic specific radioactivity following a single injection of ^{3}H-TdR or ^{125}I-UdR in leukaemic mice, in order to study the extent of the reutilization of tritiated thymidine. It has been shown that isotopic labelling with I-UdR is stable throughout the life of the cell (Hughes *et al.*, 1964) and when a suitable dose is used, ^{125}I-UdR does not alter the cell proliferation kinetics (Feinendegen, Bond & Hughes, 1966; Dethlefsen, 1974). We have confirmed these two properties of the isotope in a strain of mammary tumour cells (EMT6) *in vitro*. This system is as close as possible to our *in vivo* model and is well defined.

MATERIALS AND METHODS

Virus. The polycythaemic inducing strain of Friend virus has been maintained for 6 years in our laboratory by passage through DBA/2 or Swiss mice. The virus stock was obtained from leukaemic spleens homogenized in a PBS buffer solution containing 0·5 M saccharose. The supernatant of this homogenate, obtained after two successive centrifugations at 4000 rpm and 9000 rpm (Sorvall RC19), was filtered through a millipore filter 0·45 μ, divided into ampoules and stored at –70°C.

Mice. According to the different experiments, we have either used inbred S Swiss mice or DBA/2 mice. The animals were female, aged 2 months and they weighed about 18–20 g. Fifteen days before the experiment, 50 SD_{50} [1 SD_{50} = 1 spleen dose 50% (Rowe & Brodsky, 1959)] of the virus suspension was injected into mice intravenously in a volume of 0·2 ml.

Cells. The experiments *in vitro* were made using EMT6 cells; this line was established from a mouse mammary tumour and has been provided by Dr E. P. Malaise (Institut Gustave Roussy, Villejuif). The EMT6 cells grew in monolayer, in Waymouth medium (Eurobio-France) containing 10% embryonic calf serum (Gibco).

Radioactive compounds. ^{3}H-methyl thymidine (^{3}H-TdR) and ^{125}I-Iododeoxyuridine (^{125}I-UdR) were purchased from the Radiochemical Centre, Amersham. For the *in vivo* studies 0·5 μCi/g of ^{3}H-TdR (specific activity 5 Ci/mmole) and of ^{125}I-UdR (specific activity 6·5 mCi/mg) were injected into the retro-orbital sinus in a volume of 0·2 ml. For the *in vitro* studies ^{3}H-TdR (specific activity 25 Ci/mmole) and ^{125}I-UdR (specific activity 500–900 μCi/ml) were added to the tissue culture medium at a dose of 0·2 μCi/ml.

Study of the evolution of the labelling index of splenic cells after a single injection of ^{125}I-UdR or ^{3}H-TdR (0·5 μCi/g)

Two groups of fifty-two Swiss leukaemic mice were injected at time 0 either with ^{125}I-UdR or ^{3}H-TdR. Starting 1 hr after this injection, then 2-hourly for the next 19 hr, and then at 25 hr, 30 hr and 40 hr, four mice were killed. The spleen was removed and fixed in buffered formalin, and histological sections prepared for autoradiographic analysis. The sections were soaked in a 1% aqueous solution of glycerine, then dipped in undiluted Ilford K2 emulsion, previously warmed at 42°C for half an hour. The duration of exposure was adjusted to obtain the maximum modal number of grains per cell in the sections taken 1 hr after the injection. This was 8 weeks for animals labelled with ^{3}H-TdR and 12 weeks for those injected with ^{125}I-UdR. The percentage of labelled cells was measured by counting 500 cells per slide and four slides. All cell types in the leukaemic spleen were counted and under our experimental conditions a cell was required to have at least three grains over the nucleus for it to be considered as labelled. The modal number of grains per labelled cell was determined by counting 200 labelled cells.

Calculation of the radioactivity incorporated into 100 cells in the leukaemic spleen (specific radioactivity)

The product of the modal number of grains per cell and the percentage of labelled cells in the organ is a measure of the total radioactivity incorporated per 100 cells in the organ. The reutilization of tritiated thymidine was calculated from the turnover rates observed from DNA labelled with ^{3}H and ^{125}I (Feinendegen *et al.*, 1973).

Measurement by external counting of the evolution of specific radioactivity in the spleen of leukaemic mice after a single injection of ^{125}I-UdR or ^{3}H-TdR

Two groups of forty leukaemic DBA/2 mice were injected at time 0 with 0·2 ml solution of ^{125}I-UdR or ^{3}H-TdR. One hour after this injection and then every 2 hr for 15 hr, and then at 20 hr and 30 hr, four mice were killed; the radioactivity incorporated in the spleen was measured directly by external counting. The spleens labelled by ^{125}I-UdR were kept for 5 days in buffered formalin (Burki *et al.*, 1970). This treatment eliminated the acid soluble derivatives of I-UdR from the spleen without any loss of the molecules incorporated

into DNA. The spleens were then put into Packard flasks containing fresh formalin and their radioactivity measured by a Packard gamma scintillation counter. The spleens labelled by ^{3}H-TdR were dissolved in Soluene 350 (Packard) at 50°C and the radioactivity of the solution thus obtained was measured by a Packard scintillation counter.

Study of the effect of ^{125}I-UdR on the doubling time of EMT6 cells

Twenty-four Falcon flasks (25 cm^2) were seeded with 3×10^6 EMT6 cells. Twenty hours later up to 0·5 ml of a solution of ^{3}H-TdR or ^{125}I-UdR (0·2 μCi/ml) were each added to three flasks. After an incubation of 15 min at 37°C, the cells were washed twice and the radioactive medium replaced by 2 ml of Waymouth medium containing cold thymidine (Merck; 1 g/l) and incubated for a further 10 min at 37°C. At 20 hr, 27 hr and 42 hr, three flasks from each group were sampled as above, as well as three flasks at 20 hr and 42 hr which had not been incubated with radioactive material. The medium was discarded and the cells were rinsed with 2 ml of PBS buffer, then fixed with methanol. After it had been verified that the distribution of the cells over the surface of the flasks was homogenous, the number of cells per cm^2 was determined in ten different areas of the Falcon flask using an inverted microscope (magnification ×80) and a millimetre ocular reticule.

Study of the stability of the quantity of radioactive precursor incorporated into EMT6 cells during their exponential growth

The EMT6 cells were seeded and labelled with ^{125}I-UdR or ^{3}H-TdR as in the previous experiment. Twelve flasks were used for each radioactive precursor. The cells were then washed a sufficient number of times for the radioactivity present in the supernatant to be at maximum equal to 10% of the radioactivity incorporated in the cells. At times 0, 22 hr and 34 hr after the last wash, four flasks were taken from each group. The culture media were decanted from each flask individually and filtered through a millipore filter (0·45 μ) so as to eliminate any mitotic cells that might have been shaken off; the radioactivity of the supernatant was measured by direct scintillation counting for the ^{125}I-UdR labelling, and after the addition of 0·1 ml of the medium to 10 ml of liquid scintillator (Packard) for the ^{3}H-TdR labelling.

In parallel, the cells in monolayers were rinsed successively with 0·2 ml PBS buffer, 10% trichloroacetic acid, a solution of ethanol-ether (3 V, 1 V) and then dried. They were then harvested by scraping with 2 ml of 0·2 M NaOH; this last step was observed using the inverted microscope. The radioactivity of the solution obtained was then measured.

Study of the influence of the number of washes on the release of molecules of ^{125}I-UdR incorporated into EMT6 cells in exponential growth

EMT6 cells were seeded, under the same conditions as in the previous experiment, into eight Falcon flasks. They were incubated on the next day for 15 min with 0·2 μCi/ml ^{125}I-UdR. After this incubation, half of the flasks were washed five times and the other half ten times. At 0, 1 hr, 3 hr, and 19 hr after this washing the culture media were recovered individually and their radioactivity measured after they had been filtered through a 0·45 μ millipore filter. After each sampling the culture media were replaced by fresh media (Waymouth media with 10% embryonic calf serum) and the flasks were incubated at 37°C until the following sampling.

RESULTS

I. *Study of the effect of ^{125}I-UdR on the doubling time and the stability of labelling of cells in vitro*

The doubling time of EMT6 cells is 12·5 hr under our experimental conditions and it is not modified by the addition of 0·2 μCi/ml of ^{3}H-TdR or ^{125}I-UdR (Fig. 1).

Table 1 shows the rate of change in radioactivity incorporated by EMT6 cells in exponential growth after they have been labelled at time 0 with ^{3}H-TdR or ^{125}I-UdR. It can thus be seen that whatever isotope was used, the quantity of radioactivity bound to the cells remained constant during the 30 hr following labelling, that is approximately the duration of three mitotic cycles. However, whilst with ^{3}H-TdR labelling no radioactivity

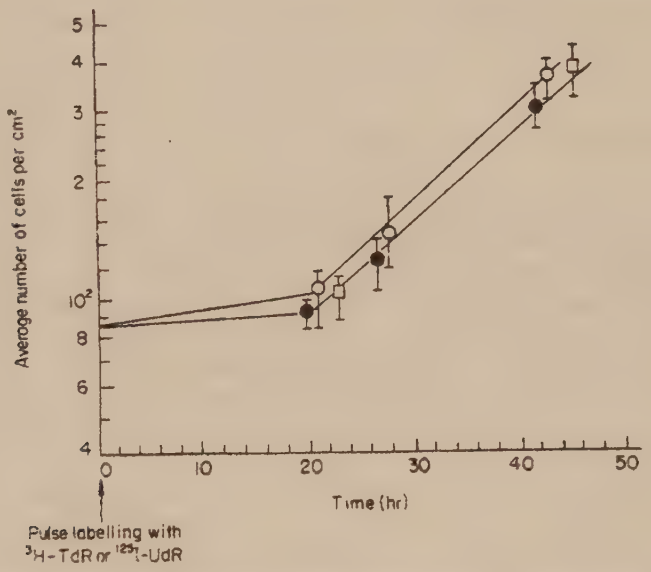

FIG. 1. Growth curves of MT6 cells after a single labelling with ^{125}I-UdR (○) or ^{3}H-TdR (●) (0·2 μCi/ml). □, No labelling.

TABLE 1. Incorporation of radioactivity into MT6 cells in exponential growth phase after a brief labelling with ^{3}H-TdR or ^{125}I-UdR (0·2 μCi/ml)

Time after labelling (hr)	^{3}H-TdR	^{125}I-UdR
0	1773 (581)	3208 (734)
20	2717 (990)	3708 (949)
30	2520 (878)	3098 (1010)

Cells were labelled for 15 min at 37°C and washed five times after they had been labelled with ^{3}H-TdR, and thirteen times after they had been labelled with ^{125}I-UdR. Each number represents the mean (cpm) of four values. The standard deviation is indicated in parentheses.

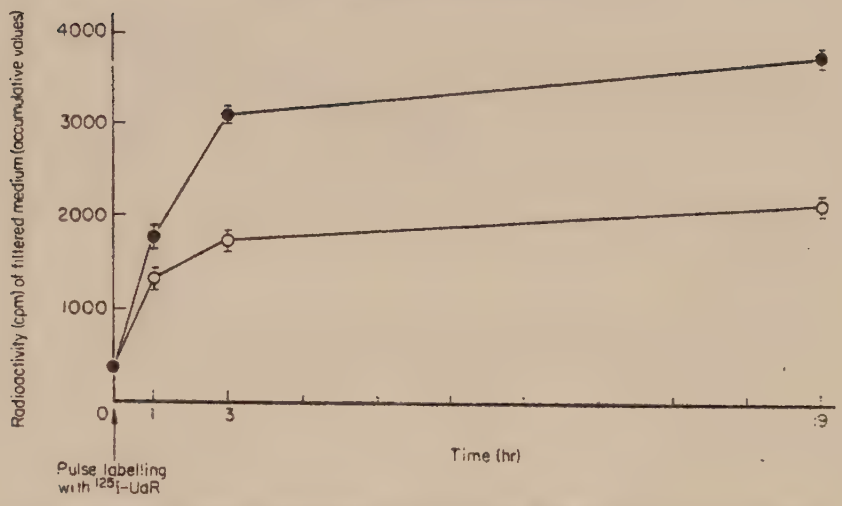

FIG. 2. Release of cellular radioactivity from MT6 cells after five (○) and ten (●) washes.

appeared in the culture medium, when ^{125}I-UdR was used as the label there was a considerable increase in the amount of radioactivity in the culture medium; this increase rose by a factor of 4, 30 hr after the labelling. But, as we have indicated previously, the cells were washed a sufficient number of times at time 0 to ensure the minimum quantity of radioactivity in the culture medium: this required five washes for ^{3}H-TdR labelling and fifteen when labelling with ^{125}I-UdR. The influence of the number of washes on the liberation of radioactivity from the cells was studied and Fig. 2 shows how the total amount of radioactivity released by the cells into the medium changed with time according to whether the cells were washed five or ten times. It was seen that washing, by virtue of its repetition, had an immediate action on the release of cellular radioactivity (from 1 hr there is a noticeable difference between the two treatments). This specific action of the number of washes is prolonged for 3 hr, from then on the cellular radioactivity continued to be liberated slowly at the same speed for both types of label until 19 hr. The increase of radioactivity in the culture medium observed in the previous experiment following the labelling with ^{125}I-UdR is probably then an artefact due to the large number of washes which probably damage some of the cells. As the quantity of radioactivity incorporated into the cells remains constant during the course of the experiment it can be concluded that the isotope is incorporated in a stable form into the cells, at least for the duration of the experiment.

II. *Change in the percentage of labelled splenic cells after an injection of ^{3}H-TdR or ^{125}I-UdR in vivo in Friend leukaemic mice*

The percentage of labelled proerythroblasts and basophilic erythroblasts in the spleen 1 hr after the injection of ^{125}I-UdR was on average 45% in both cases. After fluctuations during the first 7 hr, particularly following labelling with ^{125}I-UdR, we observed an exponential reduction of labelling indices, which was more rapid when cells were labelled with ^{125}I-UdR ($T_{1/2} = 11{\cdot}8$ hr) ($T_{1/2}$ = time required for 50% of the radioactivity to disappear from the spleen) than when ^{3}H-TdR was used ($T_{1/2} = 28$ hr) (Fig. 3). In both these experiments the modal grain count per cell 1 hr after the injection was low, being twelve and nine

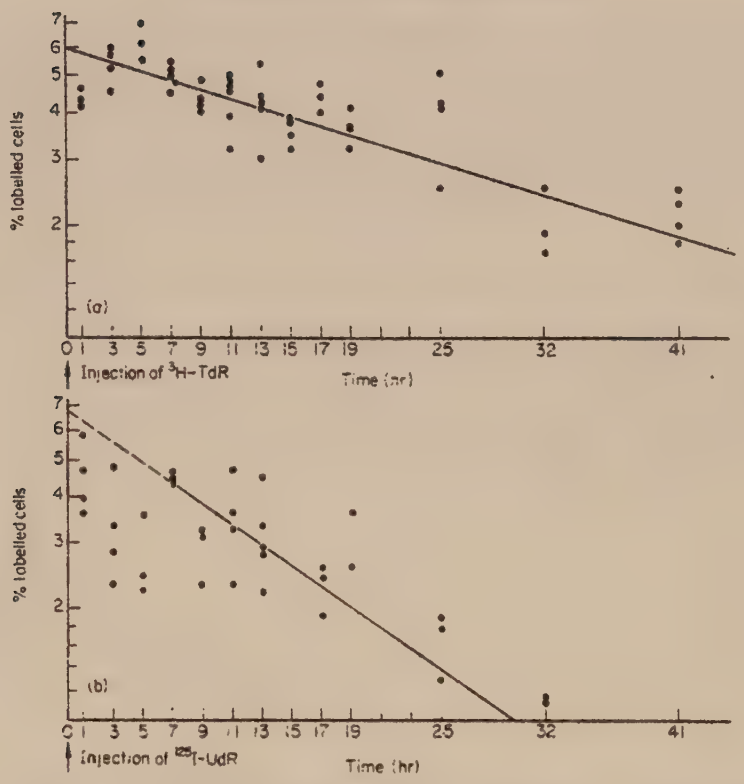

FIG. 3. Change in the percentage of labelled cells in the spleen after a single injection of ^{3}H-TdR (a) or ^{125}I-UdR (b). Each point represents individual values. The percentage was calculated on 2000 cells. Regression curves are characterized by the following parameters: ^{3}H-TdR: $r = 0{\cdot}8773$, slope $= -0{\cdot}0248$; ^{125}I-UdR: $r = 0{\cdot}9416$, slope $= -0{\cdot}0583$.

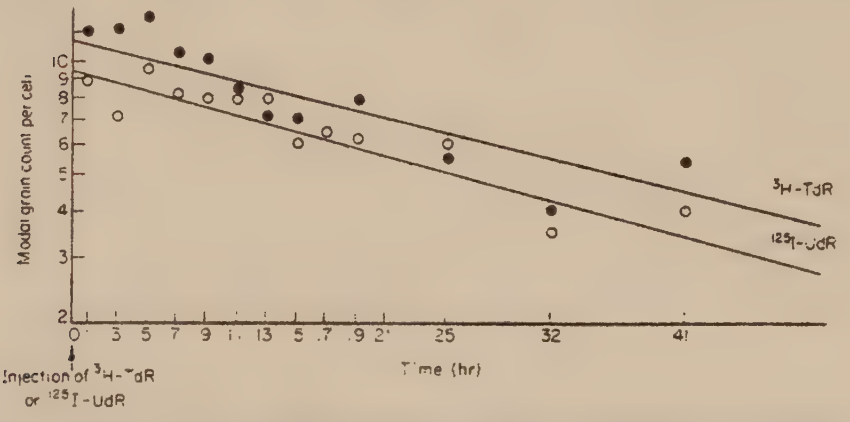

FIG. 4. Change in the modal grain count of leukaemic cells in the spleen after a single injection of ^{3}H-TdR (●) or ^{125}I-UdR (○). Each observation was made on 200 labelled cells. The regression curves were as follows: ^{3}H-TdR: $r = 0{\cdot}8997$, slope $= -0{\cdot}0272$; ^{125}I-UdR: $r = 0{\cdot}9050$, slope $= -0{\cdot}0228$.

grains for labelling with ^{3}H-TdR and ^{125}I-UdR respectively. These values diminished with an apparently similar slope in the two experiments as shown in Fig. 4 (^{3}H-TdR: $r = 0{\cdot}8997$, slope $= -0{\cdot}0272$; ^{125}I-UdR: $r = 0{\cdot}9050$, slope $= -0{\cdot}0228$) (r = correlation coefficient). But, by the twenty-fifth hour, the modal grain count was too low (respectively 5 and 6·5 for ^{125}I-UdR and ^{3}H-TdR labelling) for any difference between the slopes to be significant. During the 42 hr following the injection of ^{125}I-UdR or ^{3}H-TdR, the percentage of mitoses in the spleen remained constant and was approximately 10%.

III. *Change of specific radioactivity in the spleen of leukaemic mice after a single injection of ^{3}H-TdR or ^{125}I-UdR in vivo*

Autoradiography. The product at each time point of the modal grain count per cell and the labelling index is the radioactivity present in 100 cells (specific radioactivity). The change of this specific radioactivity during the time after injection of ^{3}H-TdR or ^{125}I-UdR *in vivo* is shown in Fig. 5. It was observed that this radioactivity decreased constantly when ^{3}H-TdR was used as the label ($r = 0{\cdot}9327$; slope $= -0{\cdot}0459$; $T_{1/2} = 15$ hr). When

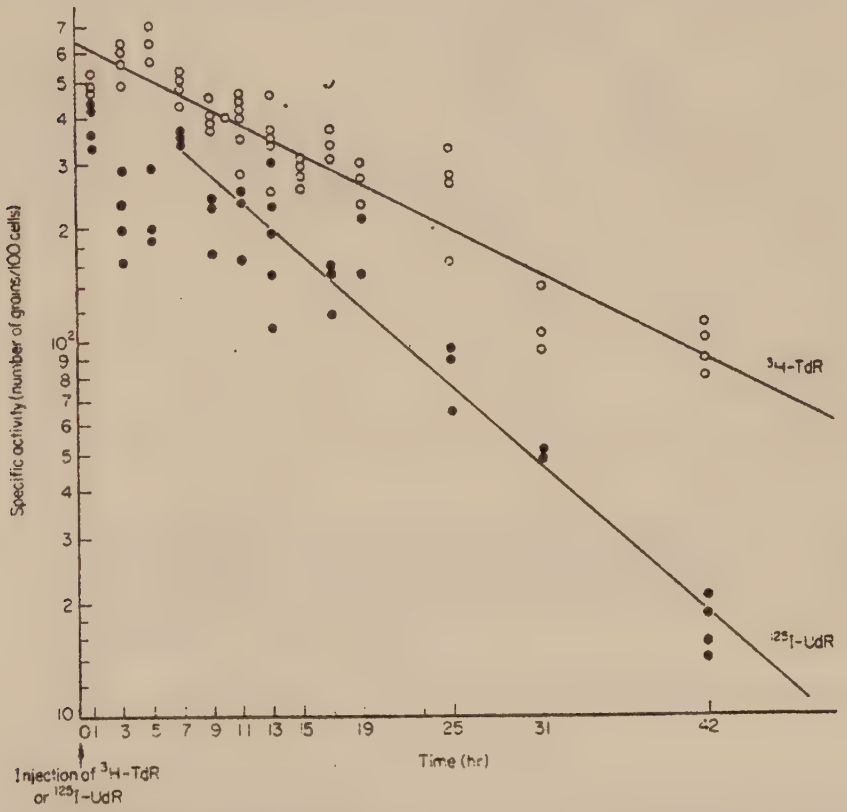

FIG. 5. Change in the specific radioactivity calculated from autoradiographic data in the leukaemic spleen after a single injection *in vivo* of ^{3}H-TdR or ^{125}I-UdR. The points represent individual values. Regression lines have the following characteristics: ^{3}H-TdR: $r = 0{\cdot}9327$, slope $= -0{\cdot}0459$, $T_{1/2} = 15$ hr; ^{125}I-UdR: $r = 0{\cdot}9618$, slope $= -0{\cdot}0821$, $T_{1/2} = 8{\cdot}6$ hr (7 hr → 42 hr).

^{125}I-UdR was used the radioactivity did not diminish in a regular fashion until after 7 hr, and then it was at a rate of approximately twice as rapid as that of ^{3}H-TdR ($r = 0{\cdot}9618$, slope $= -0{\cdot}0821$; $T_{1/2} = 8{\cdot}6$ hr).

External counting. The change of specific radioactivity expressed in cpm/g spleen after injection of ^{3}H-TdR or ^{125}I-UdR is shown in Fig. 6. Once again there was an exponential diminution in the specific radioactivity of the spleen, starting from the first hour when ^{3}H-TdR was used. The correlation between the different values is less good than seen previously: $r = 0{\cdot}6838$; slope $= -0{\cdot}0244$; $T_{1/2} = 28{\cdot}4$ hr. The specific radioactivity of ^{125}I-UdR diminished exponentially from the first hour, but at a faster rate: $r = 0{\cdot}6896$; slope $= -0{\cdot}0596$; $T_{1/2} = 11{\cdot}6$ hr. A better correlation is obtained if we consider the values from the seventh hour: $r = 0{\cdot}8333$; slope $= -0{\cdot}0485$; $T_{1/2} = 14{\cdot}3$ hr.

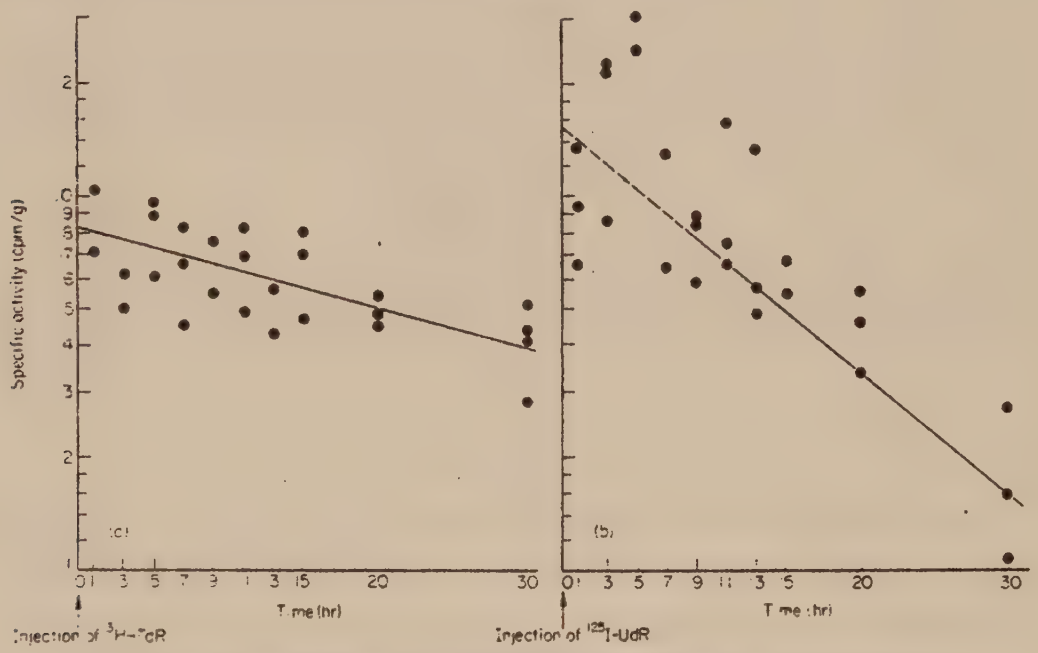

FIG. 6. Change of specific radioactivity calculated from external counting data in the leukaemic spleen after a single injection of ^{3}H-TdR (a) or ^{125}I-UdR (b). The points represent individual values. Regression curves are characterized as follows: ^{3}H-TdR: $r = 0{\cdot}6838$, slope $= -0{\cdot}0244$, $T_{1/2} = 28{\cdot}4$ hr; ^{125}I-UdR: 7 hr → 30 hr: $r = 0{\cdot}8333$, slope $= -0{\cdot}0485$, $T_{1/2} = 14{\cdot}3$ hr; 1 hr → 30 hr: $r = 0{\cdot}6896$, slope $= -0{\cdot}0596$, $T_{1/2} = 11{\cdot}6$ hr.

IV. *Quantitation of the reutilization of ^{3}H-TdR by Friend leukaemic cells and the rate of cell loss*

The modal grain count 1 hr after injection of ^{3}H-TdR was 12 and after ^{125}I-UdR it was 9. Knowing that for the same quantity of radioactivity incorporated we observed 3 times more grains when ^{125}I-UdR was used as a label compared to ^{3}H-TdR (Ertl, Feinendegen & Heiniger, 1970), we have defined by the ratio $12/(9/3) = 4$ the relative efficiency of the incorporation of ^{3}H-TdR compared to ^{125}I-UdR. That means that ^{3}H-TdR is incorporated by Friend leukaemic spleen cells 4 times more efficiently than ^{125}I-UdR. If we assume that

this degree of discrimination against I-UdR relative to TdR, is the same also for the nucleoside salvage pathway, then, for each part of I-UdR there are approximately four parts of TdR reutilized. The respective proportions α and β of ^{3}H-TdR and ^{125}I-UdR released per hour from splenic DNA are expressed by the slopes of the exponential portion of the curves representing the time evolution of the splenic specific activity. These curves (Figs. 5 and 6) exhibit simple exponential functions from the first hour following injection in the case of ^{3}H-TdR labelling, and from approximately the seventh hour in the case of ^{125}I-UdR labelling.

If we consider that the difference $\beta - \alpha$ represents the reutilization of three out of four parts TdR, then the entire amount of TdR (x) which is reutilized per hour, via the nucleoside salvage pathway, may be calculated as follows (Feinendegen *et al.*, 1973):

$$x/4 = (\beta - \alpha)/3$$
$$x = (4/3)(\beta - \alpha)$$

A coefficient γ representing the renewal rate, per hour, of ^{3}H-DNA in the absence of reutilization of free ^{3}H-TdR can be calculated:

$$\gamma - \alpha = x = (4/3)(\beta - \alpha)$$
$$\gamma = (4/3)(\beta - \alpha) + \alpha$$
$$\gamma = (4/3)\beta - (1/3)\alpha$$

Therefore, the percentage of the amount of ^{3}H-TdR that is, per hour, released from the splenic DNA and reutilized can be calculated by the following formula (Feinendegen *et al.*, 1973):

$$R = \frac{\gamma - \alpha}{\gamma} \times 100$$
$$R = \frac{1{\cdot}3(\beta - \alpha)}{1{\cdot}3\beta - 0{\cdot}3\alpha} \times 100$$

The values of γ and R calculated from the data obtained with autoradiography or external counting are summarized in Table 2.

TABLE 2. Summary of the main parameters characterizing the migration and cell loss of Friend leukaemic cells in the spleen

	$T_{1/2}$ (hr)		α	β		R = % reutilization
	^{3}H-DNA	^{125}I-DNA	^{3}H-DNA	^{125}I-DNA	γ	of ^{3}H-TdR
External counting	28	14*	0·0244	0·0485*	0·0565	56·83
Autoradiography	15	8·5	0·0459	0·0821	0·0941	51·25

$T_{1/2}$: time for the diminution by 50% of the specific radioactivity.

α and β: regression coefficients: slopes of the exponential portion of the curves representing the time evolution of the splenic specific radioactivity. They describe the hourly renewal of ^{3}H-DNA and ^{125}I-DNA respectively.

γ: hourly renewal rate of ^{3}H-TdR DNA in the absence of reutilization of ^{3}H-TdR.

R: percentage of ^{3}H-RdR reutilized by the cells.

* Value calculated from experimental data obtained from the seventh hour after ^{125}I-UdR injection till the thirtieth hour.

External counting of radioactivity and autoradiography gave slightly different percentages of reutilization but it was about 50% for both techniques. The rate of renewal of ^{3}H-TdR per hour by the cells in the Friend leukaemic spleen is more rapid (9·4% per hour) when it is calculated from the autoradiographic data than from the results of external counting (5·7%).

DISCUSSION

Using ^{125}I-UdR and ^{3}H-TdR *in vivo* labelling we have been able to demonstrate that the cellular DNA in Friend leukaemic spleens was rapidly renewed ($\gamma = 9{\cdot}4\%$ per hour), and that a massive reutilization of ^{3}H-TdR (50%) takes place in this organ.

To avoid a perturbation of the kinetics of the labelled cells, the injection of less than 0·03 μmoles of I-UdR per mouse and less than 1 μCi/g are recommended (Dethlefsen, 1974). In our experiments we only injected 0·5 μCi/g of ^{125}I-UdR; its specific activity being 6·5 mCi/mg, each mouse received 0·006 μmole of ^{125}I-UdR. The constancy of the mitotic index in the leukaemic spleen during the 40 hr following injection of either radioactive DNA precursors confirms that the doses we used are not toxic on Friend leukaemic cells. We have also ensured that ^{125}I-UdR is not liberated from DNA before cell death, by checking that it remains incorporated into the cellular DNA during several cell divisions. For this purpose, we have used an *in vitro* system, where experimental conditions can be controlled better than *in vivo* and EMT6 cells which kinetic parameters of proliferation are well known.

The specific radioactivities of Friend leukaemic spleen declines much more rapidly when the technique of measurement is autoradiography rather than when it is external counting. In fact, the disappearance of labelled cells is not only due to their loss from the organ, but also to their entry into the unlabelled compartment when their grain count falls below 3. This phenomenon, which limits the duration of our study, rapidly becomes a major factor, because under our experimental conditions the modal grain count per cell is always low. It is particularly striking when cells are labelled with ^{125}I-UdR. Such an unusually low labelling can be only partly explained by the low amount of radioactive precursors we have injected. We suggest that there may exist also a competition between labelled and unlabelled molecules for incorporation into DNA, because of the existence of an important intrasplenic pool of thymidine and derived catabolites. In support of this hypothesis is the fact that Friend leukaemic cells incorporate ^{3}H-TdR only 4 times more efficiently than ^{125}I-UdR, whereas normal bone marrow cells incorporate it about 6 times more efficiently than I-UdR (Feinendegen *et al.*, 1973). As we shall discuss later, this great pool of thymidine in the Friend leukaemic spleen may result from a continuous intrasplenic death.

The slower decline of specific radioactivity when measured by external counting may be also explained by the fact that some catabolites of ^{3}H-TdR or ^{125}I-UdR have a prolonged existence in the spleen (Fox & Prusoff, 1965; Ertl *et al.*, 1970). In our experiments, ^{3}H-labelled catabolites were counted, and this explains the great difference between autoradiographic and external counting results. On the contrary, the results are more concordant in the case of ^{125}I-UdR labelling, probably because in this case ^{125}I-labelled catabolites have been previously removed by buffered formalin (Table 2).

Autoradiography allows a more precise determination of the parameters we want to determine (rate of cellular DNA turnover, degree of ^{3}H-TdR reutilization), since only the

radioactivity incorporated into the cellular DNA is measured; however, external counting is an easier technique, which presents the advantage of avoiding the interference of background values with the experimental results, and which can rapidly demonstrate the existence of a reutilization of ^{3}H-TdR.

With each technique, the reutilization of ^{3}H-TdR was obvious (Figs. 5 and 6). According to the method of Feinendegen (1973), it was estimated to be about 50% (51% when calculated from autoradiographic data, and 58% when calculated from external counting data). This value is very high, only slightly less than for murine thymic lymphocytes, and of the same order as for normal basophilic erythroblasts (55%) (Heiniger, Feinendegen & Burki, 1971).

These molecules of ^{3}H-TdR, still present in great quantity 40 hr after injection of the isotope, can only originate from the DNA of dead cells (Feinendegen *et al.*, 1966; Cleaver, 1967). Therefore, the time at which reutilization is noticeable, at 7 hr, is also the time where the first cellular death becomes obvious. This death probably concerns mainly proerythroblasts and perhaps also basophilic erythroblasts. In fact, they are the only erythroid cells labelled 1 hr after the injection of the isotope (unpublished data); and the acidophilic erythroblasts, which are not numerous in Friend leukaemic spleen (Smadja-Joffe *et al.*, 1973), start to differentiate into anucleated reticulocytes only much later (after 24 hr in rat bone marrow) (Heiniger *et al.*, 1971).

If we consider that the decrease of the splenic specific radioactivity is a simple exponential function, the true splenic DNA turnover can be described by the following equation:

$$A_{t24} = A_0 \times e^{-\gamma t24}$$

where A_0 and A_{t24} are the splenic specific radioactivities (total labelling per 100 cells or cpm per gramme of spleen weight) respectively at time 0 and 24 hr after injection of the radioactive precursors. γ is the true hourly renewal rate of DNA in the absence of TdR reutilization. For reasons outlined earlier, autoradiographic data seem to be more exact than data obtained from external counting experiments. Therefore, using $\gamma = 9{\cdot}41\%$ (Table 2), it was calculated (see mathematical appendix 1) that 89·5% of the DNA bound specific radioactivity disappears within 24 hr. This value differs from the turnover rate (77% per day) calculated from discrepancy between cell production and spleen weight increase (see mathematical appendix 2).

Finally, most of the hyperbasophilic cells produced in the Friend leukaemic spleen die rapidly; it has been shown (Smadja-Joffe *et al.*, 1973; Tambourin *et al.*, 1973) that a minority of them is capable of accelerated maturation into red cells, this differentiation also representing a delayed death. These two important characteristics of Friend leukaemic cells argue in favour of a continuous recruitment of hyperbasophilic cells from a precursor pool, the positive balance between this cellular production and the cell loss explaining the progressive increase in the number of splenic hyperbasophilic cells. These findings also raise questions concerning the degree of malignancy of Friend leukaemia splenic cells: in fact,

they have neither the unlimited capacity of proliferation nor the long life span which often define neoplastic cells. They only show signs of a pathology which provokes their rapid death.

MATHEMATICAL APPENDICES

Appendix 1: *Calculation of the daily DNA turnover in the Friend leukaemic spleen*

The true splenic DNA turnover is described by the equation:

$$A_{t24} = A_0 \times e^{-\gamma t 24}$$

A_{24}: splenic specific radioactivity 24 hr after injection of the radioactive precursor; A_0: splenic specific radioactivity at time 0; γ: hourly renewal rate of DNA in the absence of TdR reutilization = 9·41 % when autoradiographic data are used (Table 2). For $A_0 = 100$, A_{24} can be calculated as follows:

$$\gamma_{24} = 0{\cdot}0941 \times 24 = 2{\cdot}2584$$

$$\frac{A_0}{A_{24}} = e\gamma^{24} = e^{2{\cdot}2584} = 9{\cdot}5677$$

$$A_{24} = \frac{100}{9{\cdot}5677} = 10{\cdot}45$$

That means that within 1 day the DNA bound radioactivity declines to 10·5 %, or that 89·5 % of it leaves the spleen.

Appendix 2: *Calculation of DNA turnover in Friend leukaemic spleen*

The growth of the leukaemic spleen can be described by the following equation:

$$\frac{dN(t)}{dt} = (k_p - k_1)\, N(t) \qquad (1)$$

$N(t)$: cell number at time t; k_p: rate of cell production; k_1: rate of cell loss. If we make the hypothesis that the cell loss occurs randomly in the proliferative as well as in the non-proliferative compartment, therefore, from equation (1), the following relation can be deduced:

$$\frac{dA_t}{dt} = -k_1 A_t \qquad (2)$$

A_t: splenic specific radioactivity at time t,
and

$$\frac{A_{24}}{A_0} = e^{-k_1 \cdot 24}$$

A_0 and A_{24} are the splenic specific radioactivities respectively at time 0 and 24 hr following the injection of the isotopic precursor.

If gf is the growth fraction (Mendelsohn, 1962); ϕ the cell loss factor (Steel, 1968); and T_c the duration of the cell cycle, therefore $k_1 = \phi . [k_p]$

$$k_1 = \phi . \left[\ln(1 + \mathrm{gf}) . \frac{1}{T_c} \right]$$

In an earlier publication, the following parameters have been calculated (Smadja-Joffe *et al.*, 1973):

$$T_c = 7{\cdot}6 \text{ hr} \qquad \text{gf} = 0{\cdot}64 \qquad \phi = 0{\cdot}94$$

It follows that

$$k_1 = 0{\cdot}94 \times \ln 1{\cdot}64 \times \frac{1}{7{\cdot}6}$$

$$k_1 = 0{\cdot}0611$$

and

$$\frac{A_{t24}}{A_0} = e^{-0{\cdot}0611 \times 24} = e^{-1{\cdot}4664} = 0{\cdot}2307$$

That means that 23 % of the splenic radioactivity present at time 0 remain in the organ after one day, or that 77 % of it is lost from the organ.

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1.3. Friend leukemia : a model for the physiopathology of human chronic leukemia

F. Smadja-Joffe, C. Jasmin, C. Kerdiles, and B. Klein. Europ. J. Cancer, 11, 831-840, 1975.

RESUME : La leucémie de Friend : un modèle pour la physiopathologie des leucémies humaines chroniques

Nous avons fait une revue bibliographique des données expérimentales relatives à la physiopathologie de la leucémie de Friend. L'ensemble de ces données concourt à suggérer que le rôle premier du virus de Friend est d'induire une différenciation des cellules du compartiment ERC (Erythropoietin Responsive Cells) en cellules proérythroblastiques. Celles-ci sont de courte durée de vie : les 2/3 environ meurent rapidement et le 1/3 se différencie en globules rouges. Ces mécanismes de perte cellulaire ne peuvent suffir à éliminer les cellules proérythroblastiques en surnombre, et dont la production permanente serait maintenue par deux facteurs : 1) la replication continuelle du virus dans les tissus infectés de l'hôte. 2) la production continue de cellules cibles (ERC), grâce à l'action des facteurs de rétrocontrôle. Nous montrons que ce modèle de la leucémie de Friend pourrait être en partie utilisé pour rendre compte de la physiopathologie de leucémies humaines chroniques, en particulier la leucémie myéloïde chronique.

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Friend Leukemia: A Model for the Physiopathology of Human Chronic Leukemia*

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Abstract—*We have reviewed from a critical point of view, available experimental data concerning the physiopathology of the erythroblastosis induced by the Friend virus in mice. From these data it appears that the presence of neoplasic cells, characterized by an autonomous capacity for growth, is not necessary to explain the disease. On the contrary, it seems that the main role of Friend virus is to induce a permanent differentiation of erythropoietin responsive cells (ERC) into short-lived proerythroblastic cells: most of these cells die rapidly and the others differentiate into mature red blood cells. These mechanisms of cell elimination cannot compensate for the overproduction of proerythroblastic cells, which is maintained by (1) the virus which is replicating continuously in the infected tissues of the host, (2) the continuous supply of target cells (ERC) stimulated by almost normal feed-back mechanisms of control.*

INTRODUCTION

NORMAL hemopoiesis is a well controlled process which supplies mature blood cells with finite lifespans from a pool of stem cells. The proliferative capacity of precursor blood cells and their progressive maturation into more specialized cells are two distinct mechanisms which characterize normal differentiation. If these mechanisms fail to respond to the control exerted by the organism, via humoral and cellular regulators, a leukemia may develop. A leukemia was defined by Dameshek [1] as a "generalized abnormal, neoplastic, self perpetuating proliferation (slow or rapid) of one of the leucocyte tissues, often associated with abnormal white blood cell counts, and eventually leading to anemia, thrombocytopenia and death". This definition, which is currently accepted, makes it obvious that the concept of leukemia is often associated with the existence of clones of pathological cells characterized by an autonomous and unlimited capacity to proliferate.

Clinical criteria have led to a distinction between acute and chronic leukemias. In acute leukemia, the normal hematopoietic cells are gradually replaced by a population of immature precursors, and different forms of acute leukemia have been described according to their morphological similarities with normal hematopoietic blast cells: lymphoid, myeloid, monocytoid and myelomonocytoid. These acute disorders seem to be characterized by a defective maturation of pathological cells and their spontaneous evolution results rapidly in death.

On the contrary, chronic leukemias are mainly characterized by an overproduction of differentiated cells which are not very different morphologically from normal cells, although some functional or chromosomal abnormalities have been described. Chronic leukemias have a clinical evolution slower than acute leukemias; some of them, namely the granulocytic leukemia (CGL) and sometimes polycythemia vera (PV) end in a blastic crisis followed by a

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rapid death. (The classification of polycythemia as a chronic leukemia is not accepted by all authors. However, as will be shown later, this disease has many physiopathological features in common with other chronic leukemias.)

The data concerning physiopathology of human leukemias remain partial, and often concern only the advanced stages of the disease. However, a limited knowledge of the *in vivo* kinetics of the cellular proliferation and maturation has been obtained with the use of radioactive components such as tritiated thymidine, ^{51}Chromium and ^{59}Iron; soft-gel cultures have allowed a better understanding of the physiology of proliferation and differentiation of leukemic cells, and a partial evaluation of the committed stem cell compartment [2, 3].

It appears, therefore, that a comprehensive study of the physiopathology of human leukemias requires good experimental models. However, until now, a correlation between human and animal hemopathies appeared difficult to establish, namely because of immunological, anatomopathological and etiological differences: for instance, Gross leukemia of mice resembles a lymphosarcoma more than an acute lymphoid leukemia. Many virus-induced leukemias have a short preleukemic phase and usually no blastic crisis is observed. But the lack of experimental models is also due to our poor understanding of their pathology. Friend leukemia, a murine erythroleukemia induced by an oncornavirus, appears to be the most studied. A great amount of valuable data has been accumulated in the last few years, which enables us to understand most of its physiopathological processes.

The purpose of this paper is to summarize these data and to propose a scheme which can explain the evolution of the disease. We will also compare this model with data concerning human leukemia.

Actual model of normal erythropoiesis

A model of erythropoiesis has been proposed recently by Lajtha [4]. In this model a definite number of pluripotent stem cells, whose turn-over rate is principally under their own control, undergoes a first step by differentiating into an erythroid committed cell population (ECP). The factors regulating this first step in differentiation are as yet unknown, but experimental data suggest that local influences might be involved (HIM: hemopoietic inductive microenvironment). The ECP cells are not a self-maintaining population; they are an amplifying transitional population characterized by an age structure which undergoes a maturation process. This means that daughter cells have a reduced capacity for proliferation compared to progenitor cells. The most mature cells of this population are the ERC (erythropoietin responsive cells). The ECP cells may divide 4 to 5 times in normal mice, this number being increased to 8–10 times on demand [5]. This maturation process is influenced by erythropoietin (EPO) which directly controls the differentiation of ERC into proerythroblasts and also affects their proliferation rate. EPO seems also to act on some further stages of erythroid differentiation, but during the more differentiated stages the capacity of the cells to undergo mitosis may be mainly controlled by the level of hemoglobin in the cytoplasm.

Furthermore, feed-back mechanisms probably control the first step in differentiation as well, since removal of a large number of ECP

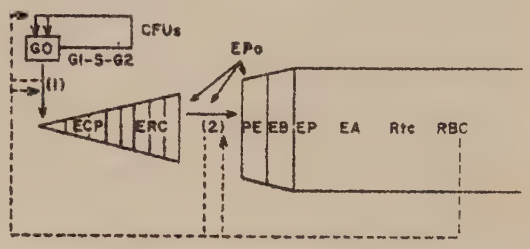

Fig. 1. Model of erythropoiesis (taken from Lajtha, L. G. [5]).

CFUs: pluripotent stem cells.
In Go: non-cycling Go state, and in G_1-S-G_2: cell-cycle.
Their proliferation is probably regulated by their own control, but their rate of turnover seems to be influenced by feed-back mechanisms.

ECP: erythroid committed population:
This population has a large amplification capacity and an age structure: ECP cells endow a maturation process, the last stage of which is the ERC (erythropoietin responsive) stage.

PE: proerythroblasts.

EB: basophilic erythroblasts (EB_1: early; EB_2: late).

EP: polychromatophilic erythroblasts.

Rtc: reticulocytes.

RBC: mature red blood cells.

$\xrightarrow{1}$: "first step" differentiation of pluripotent stem cells to erythroid committed cells. The factors controlling this step are unknown. Feed-back mechanisms and microenvironmental factors may influence it.

$\xrightarrow{2}$: "second step" differentiation: ERC cells differentiate into proerythroblasts. This step is induced by erythropoietin, which probably stimulates ERC proliferation also, and has a promoting effect on proerythroblastic maturation.

$\dashrightarrow$: Stages probably under the control of feed-back mechanisms.

EPO: erythropoietin.

into proerythroblasts apparently increases the rate of differentiation of the pluripotent stem cells, which respond to this increased stem cell removal for differentiation by an increased turnover. This model of erythropoiesis is summarized in Fig. 1, taken from L. G. Lajtha [4].

Some quantitative studies of erythropoiesis using labeling techniques with ^{59}Fe, ^{55}Fe, ^{51}Cr, ^{3}H-TdR, and cytological observations have been performed in rats [6], mice [7] and man [8]. They mainly concern the maturation process from proerythroblasts into mature red blood cells, the kinetics of proliferation and differentiation of the earlier precursor cells being less understood.

Models for the physiopathology of Friend leukemia

Friend disease is characterized by a splenomegaly (the weight of the spleen is increased by a factor of about 20), followed by an hepatomegaly and a late increase in the number of nucleated cells in the peripheral blood (from about 3000 nucleated cells per mm^3 in normal mice to more than 200,000 during the advanced stage of the disease). Anemia or polycythemia is observed according to the strain of Friend virus (FVA or FVP respectively) which has been injected, and there is an important increase in the plasma volume, which is multiplied respectively by 5 and 3 at the end of the diseases induced by FVP and FVA [9–12]. These clinical disorders are mainly due to an erythroblastosis and are detectable very early after the injection of the virus, the first pathological cells (hyperbasophilic cells, which are immature proerythroblasts) being observed in the red pulp of the spleen (under the capsule and along the trabeculae) 36 hours after injection of high doses of FVP [13]. This lag-phase is an inverse function of the dose of virus injected. The megakaryocytic and granulocytic cell lines do not appear to be perturbed by the virus, although some maturation deficiency has been demonstrated for the former [14]. Lymphocytosis has been described in spleen and peripheral blood [10], but quantitative data are still lacking. The absolute number of stem cells (CFU-s) is enhanced, especially in the peripheral blood (their number is multiplied by 800 in the polycythemic variety of leukemia) and in the spleen, where their increase parallels the splenomegaly [15, 16]. These data are summarized in Table 2.

A specific action of FV on the erythropoietin responsive cells (ERC) was suggested a few years ago [17], and recently demonstrated [18]. FVP is able to provoke the appearance of hyperbasophilic cells and splenomegaly in mice where most of the pluripotent stem cells (CFU-s) but only a few ERC have been killed with appropriate doses of myleran. Since the capacity of FV (even after multiple injections of virus) to induce leukemia is strongly reduced when the ERC population has been depleted by polycythemia in myleran-treated mice, it has been concluded that FV acts mainly by committing ERC cells to differentiate into more mature erythroid cells (hyperbasophilic cells), but it does not appear to have the capacity to restore the ERC compartment. This erythropoietin independent erythropoiesis shows strong similarities with the normal process: hyperbasophilic cells are able to mature into erythrocytes. In the spleen, erythroblast precursors are observed at different stages of their maturation [19], and the number of erythrocytes in the peripheral blood increases regularly, their total production being multiplied by a factor of about 13 at the terminal phase of the disease induced by the FVP [12]. This pathological erythropoiesis also remains sensitive, at least partially, to normal controls. Despite the fact that the virus and target cells are present in the bone marrow and in the spleen, the first hyperbasophilic cells are observed in the spleen [20], which is the main site of the pathological proliferation; furthermore, addition of erythropoietin accelerates the development of splenomegaly, and artificial polycythemia (or bleeding) delays it [21, 22].

However, several morphological and functional anomalies have been described: proerythroblasts are hyperbasophilic [20], some asynchrony between maturation of the nucleus and rate of cytoplasmic hemoglobinization is observed (personal observation), the erythropoietic maturation is only partially sensitive to normal homeostatic controls [21, 22]; but the main defect has been shown by autoradiographic studies using ^{3}H-TdR labeling which has demonstrated that hyperbasophilic cells die rapidly, in great numbers, probably after one or two mitoses [23]. This death involves at least 2/3 of the hyperbasophilic cells produced in the spleen [24].

The increase of the pluripotential stem cell compartment seems to follow the continuous depletion of the ERC compartment, and is provoked by an almost normal feed-back mechanism. Several experimental data support this hypothesis: infection of the CFU-s by FV does not confer on them a greater capacity for proliferation: infected CFU-s have an impaired ability to seed in the spleens of irradiated

normal recipients [25], the depression of the *f* factor being greater for CFU-s obtained from Friend leukemic spleen [15]. Furthermore, they remain multipotential, since splenic CFU-s transplanted into irradiated recipients are able to provide granulocytic, megakaryocytic and erythroid colonies, in the same proportion as normal [14–16]. The functional

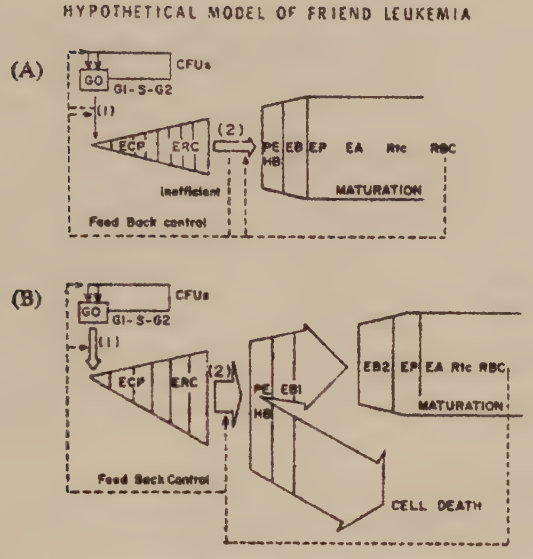

Fig. 2. *Hypothetical model of Friend leukemia.* (A) *Early action of the virus.*

The Friend virus acts mainly on the "second step" differentiation. It provokes a pathological differentiation of ERC cells into proerythroblastic cells. This differentiation escapes from the normal controls: feed-back mechanisms and EPO action. But feed-back mechanisms continue to control the "first step" differentiation[1].

HB: hyperbasophilic cells which are pathological proerythroblasts or closely related precursors.

For legends, see Fig. 1.

(B) *Pathological process of the disease (exponential growth phase).*

The Friend disease is characterized by (1) *an overproduction of proerythroblastic cells (PE or hyperbasophilic cells HB) which is mostly inefficient since at least 2/3 of the PE die rapidly and since RBC are short lived. This overproduction results in* (2) *a continuous depletion of the ERC compartment and therefore the production of precursor cells: CFUs, ECP, ERC, is stimulated, apparently, by feed-back mechanisms of control. The balance between the production of PE and their death (with or without maturation) is positive.*

For legends: see Fig. 1.

deficiencies which are observed, namely in their capacity to produce thrombocytes, may be due to the fact that these CFU-s, as all the cells of the organism [9], are infected by the FV. However, this infection does not result in a neoplasic process at the level of the CFU-s.

From all these experimental data it appears that the abnormal overproduction of erythroid cells provoking Friend leukemia may only be due to a permanent differentiation of ERC into hyperbasophilic cells. This differentiation is induced and maintained (1) by the virus which is replicating continuously in the infected tissues of the host; (2) by the continuous supply of target cells (ERC) resulting from the action of "normal" feed-back mechanisms of control. It seems, therefore, unnecessary to postulate the existence of "neoplasic" clones having an unlimited capacity of proliferation. On the contrary, Friend leukemia cells are characterized by a massive cell death, but this mechanism of cell elimination is unable to compensate for the great overproduction of erythroid cells (proerythroblastic, erythroblastic and mature red blood cells). This model of the disease is summarized in Fig. 2.

If neoplasic cells are not necessary to explain the disease, do they exist in Friend leukemia? The main characteristics of malignant cells are correlated with their unlimited potential of multiplication: *in vitro*, malignant cells are generally capable of producing colonies when they are seeded in semi-solid agar culture medium and/or multiplying permanently in a liquid nutrient medium [26, 27]. *In vivo*, they grow and provoke death when injected into non-irradiated isogenic recipients. Numerous chromosome anomalies (aneuploidy) are also currently observed [28]. None of these characteristics have been observed in Friend leukemic cells: they cannot be cultivated *in vitro*, they are usually not transplantable into isogenic non-irradiated recipients [9, 10], and no karyotype anomalies have been observed [29].

The presence of tumor cells, transplantable into non-irradiated recipients, has been described by Thomson and Axelrad [30] as appearing early after virus injection. But only morphological criteria have been invoked, and no enhanced capacity of transplantability has been demonstrated. However, several laboratories [31–33] have obtained transplantable tumors by injecting subcutaneously pieces of leukemic spleens: in these cases, the success of the graft is exceptional (frequency 0·1%) and depends on the use of special conditions, i.e., the injection of large pieces of "old" leukemic spleen. These tumor cells have often a capacity of erythropoietic differentiation when cultivated *in vitro* [34], but they differ from the Friend leukemic cells: they have chromosomal markers, they are frequently aneuploid [33] and they are indefinitely transplantable. Therefore, they appear to be true malignant clones, arising at a late stage of the disease.

Table 1. *Clinical and physiological characteristics of human acute and chronic leukemia (excluding lymphoid leukemias)*

(A) *Clinical characteristics*

		Pathological cell lines	Infiltration by leukemic cells		
			Spleen	Bone marrow	Peripheral blood
CHRONIC LEUKEMIA	Granulocytic CGL	Granulocytic	+ + +	Increase of the granulocytic line	+ + +
	Polycythemia vera PV	Erythrocytic	+ +	Increase of the erythroid line	+ + + (plasma volume increased)
	Monocytic	Monocytic	Often enlarged	+ + +	+ +
ACUTE LEUKEMIA	Erythroleukemia	Erythroblastic	+ + +	+ +	+ + +
	Myeloblastic	Myeloblasts	Often enlarged	+ + +	+ +
	Monoblastic	Monoblasts	Often enlarged	+ + +	+ +
	Myelomonoblastic	Myloblasts and monoblasts	Often enlarged	+ + +	+ +

(B) *Physiological characteristics*

		Karyotype	Labeling indices	CFUc+		
				Number	Proliferation	Maturation
CHRONIC LEUKEMIA	Granulocytic CGL	Diploid Philadelphia chromosome	Increased in the spleen	Increased in bone marrow & peripheral blood	CSA dependent	CSA dependent
	Polycythemia vera PV	Diploid	ND++	Normal or decreased; very increased in peripheral blood	CSA dependent	CSA dependent
	Monocytic	Usually diploid	ND	ND	ND	ND
	Erythroleukemia	Aneuploid (one case)	ND	ND	ND	ND
ACUTE LEUKEMIA	Myeloblastic	Aneuploid	Normal or decreased in bone marrow	Decreased	Abortive CSA dependent	Abortive
	Monoblastic	Usually diploid	ND	ND	ND	ND
	Myelomonoblastic	Diploid or aneuploid	ND	ND	ND	ND

+In man, only CFUc can be quantified by *in vitro* techniques
++ND = not determined.

Comparison with the physiopathology of human leukemias

As it has been already outlined, the physiopathology of human leukemia is poorly understood. Clinical evolution and the degree of cellular differentiation have enabled chronic leukemias to be distinguished from acute leukemias. The clinical and physiopathological characteristics of the main categories of leukemias are summarized in Table 1. We have excluded lymphoid leukemias, which are less understood than the others, mainly because the physiology of the lymphocyte remains obscure: in contrast to other hemopoietic cells the

Table 2. Comparison between the physiopathological data concerning murine Friend leukemia, human chronic granulocytic leukemia and polycythemia vera

			Friend leukemia (Polycythemic variety)		Chronic granulocytic leukemia		Polycythemia Vera	
Clinical features	Main localization of pathological proliferation		Spleen		Spleen and bone marrow		Spleen	
	Plasma volume		Increased		ND+		Increased	
Physiological characteristics of the leukemic cells	Autonomous growth (*in vitro* and *in vivo*)		—		—		ND	
	Differentiation capacity		+		+		+	
	Death		+++		ND		ND	
	Caryotype		Diploid		Diploid (Philadelphia chromosome)		Diploid	
Physio-pathological studies	Stem cells in		No. of CFUs	Dif.+++ pattern	No.++	Dif.+++ pattern	No.++	Dif. +++ pattern
		Spleen	Increased	Almost normal	ND	ND	ND	
		Bone marrow	Normal	Normal	Normal or moderately increased	Normal	Decreased	Normal
		Peripheral blood	Very increased	Almost normal	Very increased	Normal	Very increased	Normal
	Labeling index++++	Spleen	High		High		ND	
		Peripheral blood	Low		Low		ND	

+ND = Not determined
++Number of CFUc
+++Diferentiation pattern
++++Percentage of labeled cells one hour after ^{3}H-TdR injection.

lymphocyte is a morphologically differentiated cell which can transform into a lymphoblast, and very few data regarding the physiology of this phenomenon are available. A distinction between leukemias of the hematopoietic and the immunopoietic systems is also justified by the probable existence of different stem cells for the two systems in the adult organism [4].

From this table, it appears that many physiopathological parameters have not been studied. However, available data on chronic leukemias, mainly granulocytic leukemia and polycythemia vera, and to a certain extent erythroleukemia (its classification is still a subject of controversy), have concordant features. All stages of the differentiation of the pathological cell line are found in the bone marrow, the spleen and the peripheral blood. These cells usually have a normal morphology and physiology: in the case of CGL, observations on cyclic granulocyte production [35] provide indirect evidence for the existence of positive and negative regulatory influences. The sensitivity of leukemic cells to the inhibitory effect of the granulocytic chalone has been reported [36]. In polycythemia vera it has been shown that erythropoietic differentiation was stimulated, namely in the spleen, and was accompanied by an increased plasma volume [37].

However, anomalies of the maturation process have been described: asynchrony of maturation of the nucleus and the cytoplasm in CGL and polycythemia vera [38, 39], and decreased levels of leucocyte alkaline phosphatase in CGL [40]. Cellular death has not yet been studied, but in CGL, an increase of the proportion of early precursor cells (myeloblasts, promyelo-

cytes, myelocytes) has been observed in the bone marrow of patients in relapse [41], and perhaps hyperuricemia, which is frequently observed [40], may be correlated with cell death. Defects of other cell lines usually accompanied the proliferation of the pathological cells.

There are no definite signs of malignancy: a diploid caryotype has always been found. An anomaly of the 22nd pair of chromosomes (Philadelphia chromosome) is associated with 95% of CGL [42], but whether this anomaly represents a malignant stigma is not known. However, it should be pointed out that this anomaly persists during the clinical and hematological remission of the disease, and is also found in erythroblastic and megaracaryocytic cells which are not believed to be malignant. With the exception of one report in the literature [43], and in spite of repeated attempts, the Philadelphia chromosome is lost from bone marrow and blood cells from CGL when cultured in liquid medium [44]. Peripheral blood of patients with CGL not in remission was transfused into patients with various states of leucopenia: the presence of Ph^+ cells in the bone marrow of these patients has been reported, but this "graft" has always been transient, and no induction of CGL has been reported using this procedure [45].

In the two chronic leukemias which have been the best studied (CGL and PV), the total number of CFU-c is increased as compared to normal values, namely in the spleen (CGL) and peripheral blood (CGL and PV) [39, 46]. These CFU-c keep normal characteristics of maturation and proliferation in soft-agar culture medium, and the presence of colony stimulating factor (CSF) is necessary [47].

The labeling indices (LI) observed one hour after *in vivo* injection of ^{3}H-TdR have been measured in the spleen of CGL: in this instance, high values were found, which correlated well with the stage of the disease. On the contrary, LI of the bone marrow has no indicative value [48].

Therefore, all these data concerning CGL, although incomplete, are compatible with the Friend model as shown in Table II. The most striking similarities are: absence of autonomous proliferation of the leukemic cells, the existence of a quasi-normal pattern of differentiation, an important increase in the number of committed stem cells whose capacity for proliferation and differentiation *in vitro* remain normal and CSF dependent, and privileged localization of the pathological proliferation in the spleen. A study of the cell death in CGL remains necessary to demonstrate the value of this model. In addition, it should be mentioned that Rauscher leukemia, which is an erythroleukemia almost indistinguishable from Friend leukemia, may end like CGL in blastic crisis, a myeloblastic or lymphoblastic leukemia occurring in the bone marrow [49, 50].

The clinical picture is very different in acute leukemias. There is a failure of differentiation in the pathological cell line; aneuploidy has usually been found in AML [28], the number of CFU-c is normal or decreased and there is an *in vitro* defective capacity for proliferation and maturation when seeded in soft-agar medium, although the CFU-c remain apparently dependent on CSF [51]. It is not known whether the leukemic cells can grow in liquid medium because in culture they lose distinctive features [52]. Low labeling indices have been regularly found, and abnormal cells generally have a prolonged cell cycle time [53, 54]; but most of these kinetic data are difficult to interpret since they show the existence of dividing and non-dividing cells (in AML) whose proportions vary as a function of the patient and the stage of the disease.

It is obvious that a model for studying the physiopathology of acute leukemia is needed because of the limitations of investigations in human patients. It is striking that models of myeloblastic leukemias have never been studied *in vivo*. This could perhaps be explained by the experimental difficulties due to a long latency period, and mainly because this type of leukemia was difficult to reproduce regularly in mice. The recent availability of a purified strain of myelogenous leukemia-inducing virus [55] will perhaps provide an appropriate model for the study of the physiopathology of human acute myeloblastic leukemia.

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2. Cinétique de la prolifération cellulaire dans la rate de souris DBA_2 infectées par le MPSV

2.1. Cellular proliferation in the spleen of DBA_2 mice infected with a Myeloproliferative Sarcoma Virus (MPSV)

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RESUME : La cinétique de la prolifération cellulaire a été étudiée dans les rates de souris DBA_2 infectées par un virus sarcomatogène induisant un syndrôme myéloprolifératif (MPSV). Cette étude a été réalisée à trois phases de la croissance de la rate : la phase de croissance rapide (dite "exponentielle"), la phase de croissance lente et la phase de régression.

En utilisant l'injection de thymidine tritiée *in vivo* et l'autoradiographie, nous avons montré que la majorité des cellules blastiques prolifèrent rapidement (le cycle cellulaire est égal à 10 h), même pendant la phase de croissance lente de la rate. Nous avons mis en évidence une perte cellulaire massive et rapide pendant la phase de croissance rapide de la rate (facteur de perte cellulaire $\emptyset$ = 80%). En comparant la diminution de l'activité spécifique de la rate après injection *in vivo* de ^{3}HTdR et d'^{125}IUdR, nous avons pu montrer l'existence d'une importante réutilisation de l'^{3}HTdR (R = 57%), ce phénomène étant détectable moins de 7 heures après l'injection de précurseurs radioactifs de l'ADN. Ainsi, les cellules spléniques des souris présentant le syndrôme myéloprolifératif induit par le MPSV, ont, dans leur majorité, une courte durée de vie : presque la moitié d'entre elles mature en érythrocytes et les autres meurent rapidement *in situ*.

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Cellular Proliferation in the Spleen of DBA/2 Mice Infected with a Myeloproliferative Sarcoma Virus (MPSV)

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Cellular proliferation kinetics were investigated in the spleens of DBA/2 mice infected with a myeloproliferative sarcoma virus (MPSV) at three distinct phases of spleen growth: the rapid-growth phase, the slow-growth phase and the regression phase.

Using injection of tritiated thymidine in vivo and autoradiographic techniques, we showed that most blast cells proliferate rapidly (cell cycle is equal to 10 h) even during the slow-growth phase. Very massive and rapid cell loss was found during the rapid-growth phase (cell loss factor ϕ = 80%). By comparing the decrease in specific splenic activity after in vivo injection of ^{125}IUdR and ^{3}HTdR, we were able to show a large reutilization of tritiated thymidine (R = 57%), visible less than 7 h after injection of isotopic DNA precursors. Thus, MPSV-infected spleen cells were shown, for the most part, to be short-lived cells: almost one-half mature into erythrocytes and the others rapidly die in situ.

Key words: cellular proliferation – myeloproliferative syndrome – MPSV virus

The Myeloproliferative Sarcoma Virus (MPSV) induces a myeloproliferative syndrome in DBA/2 mice whose natural history has been previously described (1,2). Cellular proliferation kinetics of this disease were investigated with two main purposes in mind. The first, to contribute a better understanding of the pathophysiology of this neoplasia; indeed, cytokinetic data have been very helpful in understanding several virus-induced leukemias which were also characterized by a large splenomegaly (3–5). The second, to determine wheth-

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er or not the cytokinetic pattern of MPSV-infected spleen cells is similar to that of Friend and Rauscher leukemias, characterized by a very rapid turnover rate or, inversely, whether the proliferation of these cells is much slower and characterized by a lower cell loss as in most solid tumors (6). Indeed, although MPSV virus provokes a considerable proliferation of hemopoietic cells, as do Friend and Rauscher viruses, the clinical aspect of an MPSV-infected mouse at a late stage of the disease evokes a malignant lymphoma or a solid tumor: the spleen is whitish and firm, and tumor nodules are observed on the peritoneal membrane. Therefore, in its clinical aspect, MPSV induced neoplasia is very different from Friend and Rauscher leukemias, which are characterized by a red spleen and lack of tumor nodules.

We determined the following: a) The growth characteristics of the leukemic spleen considered as a tumor, and b) the cytokinetic parameters of the spleen cells, since the spleen appears to be one of the main organs involved in the leukemic process (1). This study was performed at different stages of spleen growth.

MATERIALS AND METHODS

Mice. Adult DBA/2 male mice about 2 months old and weighing approximately 25–30 g were provided by Iffa-Credo (Institut Merieux, France). They were specific pathogen-free mice and were free of reovirus and Sendai virus.

Virus. The MPSV virus was prepared as described (1). The viral preparation was diluted ten times, filtered successively on 0.45 μ and 0.22 μ Millipore filter membranes and injected intravenously into the mice.

Growth curve of spleen and number of nucleated cells in peripheral blood in MPSV-infected mice. This study was performed for 1 month following intravenous injection of the MPSV virus. Seven mice were sacrificed weekly, a sample of peripheral blood was obtained by intracardiac puncture and the number of nucleated cells/mm^3 was determined. Mice, spleens and livers were weighed. Results were expressed with semi-logarithmic coordinates.

Cytological study of the neoplastic spleens. This study was performed 16, 24 and 35 days after injection of the virus. The cytologic formulas of neoplastic spleen and non-infected normal spleen were determined on smears obtained from cytocentrifuged cell suspension (Shandon-Cytospin) and stained with orthodianizine-Giemsa. A minimum of 1000 cells were observed for each spleen. To avoid a possible technical artifact due to the preparation of the cell suspension, we also determined the proportions of blast cells and mature cells (including lymphocytes) on 3 μm thick histologic sections (6000 cells were examined).

Autoradiographic study. Into MPSV-infected mice, 0.5 μCi/g of ^{3}HTdR (CEA France, specific activity 25 Ci/mmole) was injected intravenously. For a period of 20 h after this injection, at 0.5, 1, and thereafter every 2 h, four mice were sacrificed and weighed. The spleens were removed, weighed and fixed in formalin (pH 7). Histologic sections (3 μ) were cut. Tissue on one slide was stained with hematoxylin-eosin and two on other slides were autoradiographed using K5 Ilford autoradiographic gel emulsion. Exposure time at 4°C was 3 weeks and autoradiograms were developed using the gold latensification technique (7). Slides were immersed in 70% ethanol for 2 h and then stained with hematoxylin-eosin. Under these experimental conditions, the median number of grains/labeled cell was 35. Two grains or less localized on the nucleus was considered as background.

The labeling index (% of labeled cells 1 h after injection of ^{3}HTdR) and the mitotic index were determined on histologic sections by observing at least 4000 cells/spleen, excluding mature non-proliferating cells such as metamyelocytes, polymorphonuclear cells, acidophilic erythroblasts, and small lymphocytes. The % of labeled mitoses was calculated by examining at least 200 mitoses. The duration of the mitotic cycle and its distinct phases were determined from the PLM curve[1] (8), and by

[1] Curve representing the time-dependence of the % of labeled mitoses.

the optimization procedure described by A. J. Valleron (9). Indeed, since the level of labeled mitoses was more than 50%, manual interpretation of the PLM curves was impossible. According to Mendelsohn (10) the growth fraction (GF) was calculated using the formula GF = $\frac{\text{exp LI}}{\text{cal LI}}$, in which "exp LI" is the experimentally measured labeling index and "cal LI" is the theoretical labeling index of the proliferating cells, equal to $2\left[\frac{TG_2 + T_M}{T_c}(2^{\ln 2/\phi} - 1)\right]$. The cell loss factor ϕ was calculated according to Steel (11): $\phi = \frac{k_l}{k_p}$, in which k_l is the rate of cell loss and k_p the rate of cell production. k_p is equal to $\frac{\ln(1 + GF)}{T_c}$; k_l is deduced from the relationship $k_l = k_p - \frac{\ln 2}{DT}$ in which DT is the experimental doubling time of the spleen.

Specific radioactivity after a single injection of ^{125}IUdR or ^{3}HTdR during rapid growth phase of the spleen. At time 0, two groups of 40 DBA/2 mice infected 17 days before with MPSV virus were injected intravenously with 0.2 μCi/g of ^{125}IUdR (Radiochemical Center, Amersham, specific activity 7 mCi/mmole) or ^{3}HTdR (CEA France, specific activity 25 Ci/mmole). Four to five mice were sacrificed 1, 5, 10, 20, 35 and 45 h after this injection and the spleens were cut into two pieces after weighing. One piece was used for measuring the specific activity/gram of tissue, as described (4). Activity/μg of DNA was measured in the second piece. Cellular DNA was extracted by homogenizing the tissue in sodium dodecyl citrate buffer (pH 7) and nucleic acids were isolated from the tissue homogenate by precipitation in 2.5 M perchloric acid at 70°C for 2 h. The amount of DNA was determined using Burton's assay method (12) and its radioactivity was determined by liquid scintillation spectrometry. The data were expressed as % of radioactivity determined one hour after injection. Splenic specific activity curves as a function of time were plotted on semi-log coordinates and the exponential regression curves drawn were characterized by their slope α and β after ^{3}HTdR and ^{125}IUdR injection, respectively. The % (R) of tritiated thymidine continuously released from the DNA of dying cells and reutilized by the proliferating cells was calculated according to Feinendegen et al. (13).

RESULTS

Growth curves and cytologic study

Growth curves and cytologic formula of the spleen. The growth of the MPSV-infected spleen can be considered as composed primarily of three successive phases (Fig. 1). The rapid-growth phase lasts until approximately day 19 and is characterized by a doubling time (DT) of 4.3 days. At the end of this phase, mean spleen weight reaches 1250 ± 200 mg, compared to 75 ± 8 mg for controls. Organ weight thus increases by a factor of 16. In the second phase or "slow-growth" phase, the growth rate decreases. Between days 19 and 25, the mean DT measured was found to be 12.5 days. The third phase is called the regression phase. It occurs after day 27, when spleen weight decreases rapidly, reaching a mean of 400 mg at day 35, at which time the first deaths occur. The half-time of this decrease is 6.8 days. The number of viable cells/mg of spleen did not differ significantly during the three different phases of spleen growth (Table 1). In the pathologic spleen, erythroid plus myeloid cells constituted 70% of the cell population, whereas in control spleens, both populations represented no more than 23% of the cells. The proportion of erythroid cells remained roughly constant (around 45%) during the course of disease, whereas the myeloid cell population increased significantly from 22% at the exponential phase to 38% at the slow-growth and regression phases. This seems to result primarily from an increased percentage of mature myelocytes and polymorphonuclear cells. The number of lymphoid cells increased in absolute value until the slow-growth phase was reached. No more than 7% of undifferentiated blast cells, reticular

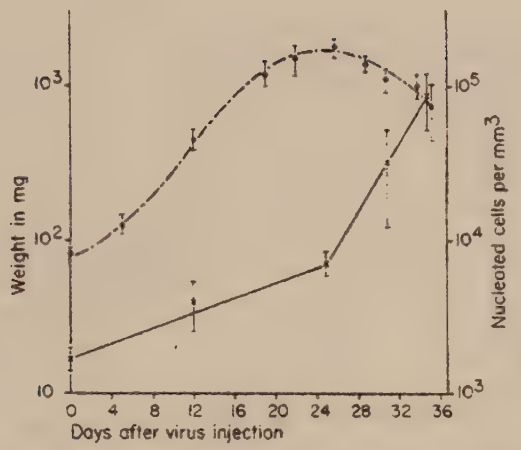

Figure 1. Time dependence of spleen weight (○——○) and number of nucleated cells in the peripheral blood (×——×) after MPSV virus injection. Each point represents the mean calculated from 7 mice.

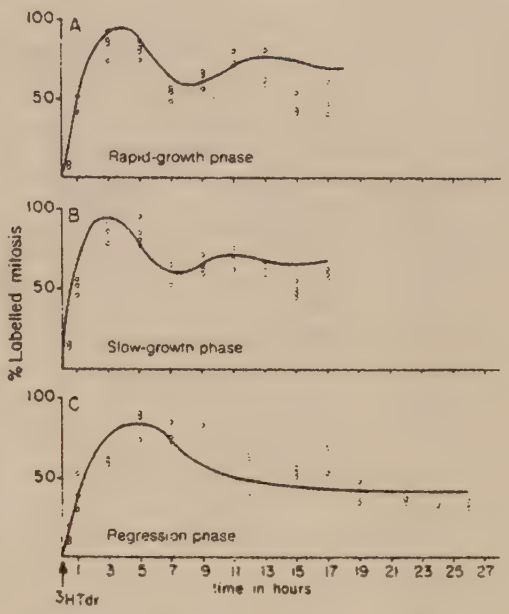

Figure 2. Curves of percentage of labeled mitosis (PLM curves) of MPSV infected spleen cells, optimalized with a mathematical model at 3 distinct phases of spleen growth. 2A: exponential phase; 2B: slow-growth phase; 2C: regression phase. Tritiated thymidine (0.5 μCi/g) was injected at time 0. Counts were performed on at least 200 mitoses for each point.

cells and monocytes could be found in smears of pathological spleen suspensions. The percentage of young blasts (32%) and of differentiated cells plus small lymphocytes (60%) was similar with both techniques (histologic and cytologic).

Body weight changes with time. A marked decrease of the body weight of infected mice was observed after day 31 and at the time of death mice weighed 18.5 ± 1.2 g, compared to 28.6 ± 1.5 g at day 0.

Number of circulating nucleated cells in peripheral blood after MPSV infection (Fig. 1). The number of nucleated cells in peripheral blood increased slowly during the first twenty-five days following viral injection. At day 24, which corresponds to the beginning of the regression phase of the spleen, the concentration of nucleated cells in peripheral blood abruptly increased, reaching $6 \times 10^4 \pm 3 \times 10^4$ cells/mm^3 (compared to 2×10^3 cells/mm^3 in peripheral blood of normal mice). Previous studies (1) have shown that 90% of these nucleated cells are polymorphonuclear cells, metamyelocytes, polychromatophilic and acidophilic erythroblasts.

Rapid-growth phase

Parameters of cellular proliferation (autoradiographic study). The PLM curve (Fig. 2A) shows a first peak at 4 h which reached 95% and then a second peak at 13 h reaching 75%. The duration of the mitotic cycle and of its various phases, as well as the major cytokinetic parameters were determined and are summarized in Table 2.

TABLE I

Cytological formulae of normal and MPSV-infected DBA_2 spleens

Days after virus injection		Normal mice	MPSV-infected mice			
		0	16 Exponential phase	24 Slow-growth phase		35 Regression phase
Mean spleen weight (mg)		75 ± 14	850 ± 200	1437 ± 294		746 ± 290
Cell number/mg (10^{-3})		11	11	9		9
Technique used		Smears	Smears	Smears	Histology	Smears
Erythroid cells	PE and BE	6.8	30.0	21.0		22.3
	PcE and AcE	5.8	19.3	11.0	31.0	19.0
Lymphocytes		76.5	22	20.0		16.5
Myeloid cells	MyeB + ProM + Myel.	1.7	5.9	7.0		5.3
	MetaM + PMN	5.4	15.9	31.0	32.0	31.0
Other cells	Monocytes	2.0	2.2	1.4		1.6
	Reticular cells	1.3	4.3	5.1		1.2
	Undifferentiated blast	0.55	0.4	3.5		2.0
	Fibroblasts	0.0	0.0	0.0	5.3	1.1
Blast cells (total)		12.3	42.8	38.0	37.0	33.5

PE: Proerythroblasts
BE: Basophilic erythroblasts
PcE: Polychromatophilic erythroblasts
AcE: Acidophilic erythroblasts
MyeB: Myeloblasts
ProM: Promyelocytes
Myel.: Myelocytes
MetaM: Metamyelocytes
PMN: Polymorphonuclear cells

"Blast" cells: include all cells except PcE, AcE, MetaM, PMN and lymphocytes.

^{3}HTdR reutilization (R) and renewal rate of the splenic DNA (γ) (Biochemical technique). The specific activity of the spleen decreased from the time of the first measurement after a single injection of ^{3}HTdR and decline can be represented by straight lines on semi-log plots (Fig. 3). The data are well correlated (correlation coefficients higher than 0.85) and in good agreement, regardless of the technique (Table 3) (Appendix 1). The specific activity after ^{125}IUdR labeling decreased more rapidly than after ^{3}HTdR labeling ($T_{\frac{1}{2}}$ = 13.4 h and 28.4 h, respectively). Tritiated thymidine was incorporated 7 times more efficiently than ^{125}IUdR (Table 4). The calculation shows that proliferating cells reutilize 57% of the tritiated thymidine released by the dead cells (Table 3).

Slow-growth phase. The PLM curve (Fig. 2B) shows a first peak at 3 h with a maximal value of 95% and a second peak which reaches 70% at 11 h. Data characterizing the cellular proliferation at this phase of the disease are summarized in Table 2.

Regression growth phase. The PLM curve shows only one well-defined peak, at 5 h, with a maximal value of 75% (Fig. 2C). The use of a mathematical model (30) was therefore necessary in order to interpret these data. Results are shown in Table 2.

DISCUSSION

Our results show that the proliferation of MPSV-infected spleen cells is char-

TABLE 2

Cytokinetic parameters of MPSV-infected spleen

Number of days after virus injection	Mean spleen weight (mg)	DT (h)	Growth phase	T_c (h)	T_{g1} (h)	T_s (h)	T_{g2} (h)	T_M (h)	LI (%)	MI (%)	GF (%)	ϕ (%)	k_p	k_l
16	850 ± 200	104	Rapid-growth	10.3	1.0	7.5	1.3	0.5	38	3.3	51	83.5	0.0469	0.0402
				(4.8)	(0.0)	(3.5)	(1.3)	(0.0)	(3)	(0.5)				
25	1435 ± 294	300	Slow-growth	9.7	1.4	7.0	0.8	0.5	43	2.6	56	95.0	0.0484	0.0464
				(6.0)	(1.7)	(3.5)	(0.8)	(0.0)	(5)	(0.6)				
33	673 ± 127	$T_l \rightarrow$ 156	Regression	31.5	15.0	14.0	2.0	0.5	41	3.4	107	107.0	0.0612	0.0657
				(31.3)	(15.0)	(14.0)	(2.3)	(0.0)	(3)	(1.1)				

DT: Doubling time
T_c: Cell cycle duration
T_{g1}: Duration of the presynthetic phase
T_s: Duration of the DNA synthesis phase
T_{g2}: Duration of the post-synthetic phase
T_M: Duration of the mitotic phase
Standard deviation: under parenthesis

LI: Labeling Index
MI: Mitotic Index
GF: Growth Fraction
ϕ: Cell loss factor
k_p: Hourly rate of cell production
k_l: Hourly rate of cell loss

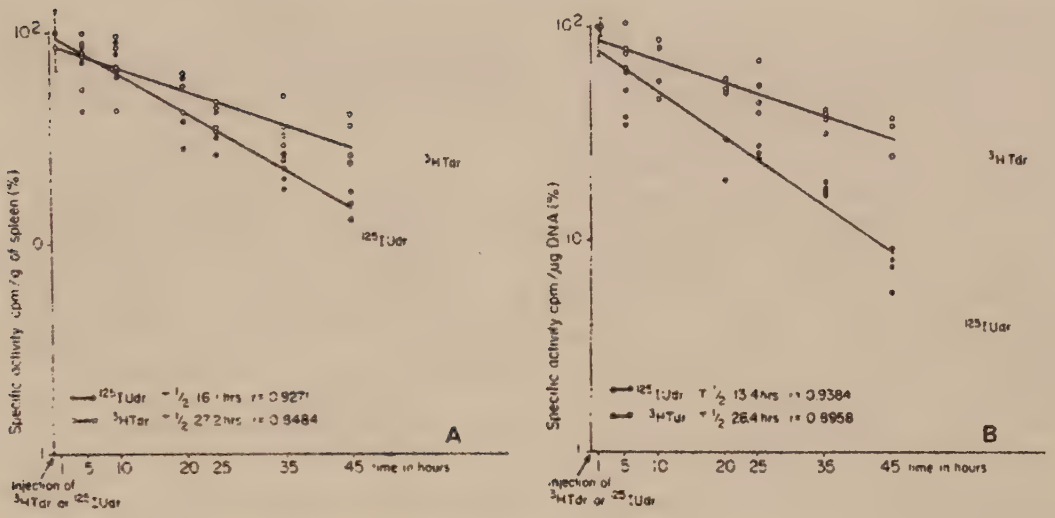

Figure 3. Specific radioactivity in MPSV-infected spleen after a single injection of ^{3}HTdR (○ ○) or ^{125}IUdR (● ●). The points represent individual values, expressed as % of those measured 1 h after injection of radioactive isotopes. A) Specific activity is measured as cpm/gram of spleen. Regression curves are characterized as follows. ^{3}HTdR: r = 0.8484, slope = −0.0255, $T_{\frac{1}{2}}$ = 27.2 h; ^{125}IUdR: r = 0.927, slope = −0.0430, $T_{\frac{1}{2}}$ = 16.1 h. B) Specific activity is measured as cpm/µg of DNA. Regression curves are characterized as follows. r = 0.8958, slope = −0.0519, $T_{\frac{1}{2}}$ = 13.4 h.

acterized during the rapid-growth phase of spleen growth by a short mean cell cycle (Tc = 10 h); however, it is not shorter than that of normal hemopoietic blasts. This value is not significantly modified during the slow-growth phase. In contrast, Tc reaches 31.5 h at the regression phase of the spleen. This lengthening is primarily due to an increase of T_{G1}, which augments by a factor of only 10, and also to an increase of Ts, which increases only twice. At the regression phase, the spread of the cell cycle time from cell to cell is also

TABLE 3

Major parameters characterizing DNA turnover rate during rapid-growth phase of the spleen in MPSV-infected DBA$_2$ mice

Splenic specific radio-activity	$T_{\frac{1}{2}}$(h)					
	^{3}H-DNA	^{125}I-DNA	α ^{3}H-DNA	β ^{125}I-DNA	γ	R (%)
cpm/g	27.17	16.13	−0.0255	−0.0430	−0.0459	44.46
cpm/µg DNA	28.36	13.35	−0.0244	−0.0519	−0.0564	56.80

$T_{\frac{1}{2}}$: Time for splenic specific radioactivity to decrease by 50%.

α and β: Regression coefficient: slopes of the exponential portion of the curves. They describe the hourly renewal of ^{3}H-DNA and ^{125}I-DNA, respectively.

γ: Hourly renewal rate of ^{3}H-DNA in the absence of reutilization of ^{3}HTdR.

R: Percentage of ^{3}HTdR reutilized by the cells.

TABLE 4

Relative efficiency of incorporation of ^{3}HTdR compared to ^{125}IUdR by MPSV-infected spleen cells

Injected isotope	A: Per g body weight	A: Per mouse	B	B/A × 100	f
^{125}HTdR	0.2	5	24×10^{-6}	4.8×10^{-4}	7
^{125}IUdR	0.2	5	3.6×10^{-6}	0.72×10^{-4}	

A: Total amount of injected radioactive isotope (μCi).

B: Amount of radioactive isotope incorporated in DNA 1 h after injection (μCi/μg DNA).

(B/A) × 100 represents the percentage of injected radioactive isotope incorporated into DNA 1 h after injection.

f: Relative efficiency of incorporation of ^{3}HTdR compared to ^{125}IUdR.

much wider than in the preceding phases. Similar behavior of the Tc parameter has been frequently observed in experimental tumors (11).

We have indeed considered the spleen as a solid tumor, and have measured its growth curve. This comparison is to be discussed, since the spleen is connected directly to the circulatory system and is thus the site of continual exchanges with cells of the blood and of other hematopoietic organs. It is nevertheless to be noted that histological examination reveals the first signs of neoplastic proliferation at the level of the spleen: furthermore, the spleen appears to be the greatest localization of the disease throughout its entire course, to the extent that the weight of this organ increases 14 fold after 28 days of evolution. In addition, the number of nucleated cells in the bloodstream appears to represent only a small proportion of neoplastic cells, since they appear only at a later phase of the disease. Finally, blast cells which are capable of dividing represent at this time only 10% of nucleated cells in the bloodstream.

The cell loss factor is very high beginning at the exponential growth phase (ϕ = 83%) and, as is the case in most experimental tumors (11), it progressively increases during the growth of the neoplastic spleen. Furthermore, the weight of the MPSV-infected spleen decreases and, at this regression phase, the cell loss factor is by definition, higher than 100%. The hourly rate of cell loss (k_l) is also very high because of the short cell cycle and the elevated Growth Fraction (Table 1). Another independent technique used to measure the rate of DNA turnover in the rapid-growth phase (see γ values, Table 3) suggests that the rates of cell loss rate and DNA turnover are in agreement.

In contrast, the time-dependences of the GF and of the rate of cellular production (k_p) are unusual. Both parameters increase progressively during the course of the disease, particularly in the regression phase of the spleen, when the GF reaches 100%. It must be mentioned that even if the GF is calculated using another formula currently used $\left(GF = \frac{LI}{Ts/Tc}\right)$ (10), a similarly high value is found in the regression phase (GF = 90.3%). This explains why, in spite of an important lengthening of Tc at this phase, the rate of cell production (k_p) is enhanced, reaching 6% per hour.

Finally, a relatively high LI which remains constant during the three stages of spleen growth reflects distinct cytokinetic patterns. In the rapid-growth, it results from the rapid proliferation of about half of the spleen cells, while in the regression phase it results from the lengthening of the Ts phase.

A striking feature of the MPSV-in-

TABLE 5

Summary of kinetic data obtained for three virus-induced murine leukemias

	Organ	Cytology of proliferating cells	Stage of disease	DT (days)	T_c (h)	LI %	GF %	ϕ	R	k_l	Ref.
Normal hematopoietic	Bone marrow	Blasts	—	—	7–8.5	56	86				5, 15
	Spleen	Blasts	—	—	—	40		100	16	0.058	5
Friend leukemia	Spleen	Proerythroblasts and basophilic erythroblasts	Exponential	6	7.6	45	64	94.0	57	0.0612	3, 4
			Plateau	18	8	30	75	98.0	—	0.0686	3, 4
Rauscher leukemia	Bone marrow	Proerythroblasts and basophilic erythroblasts	—	—	8	56		—	—	—	—
	Spleen		—	—	8	51	85	96.0	44	0.046	5
MPSV myeloproliferative syndrome	Spleen	Erythroid, myeloid and undifferentiated blasts	Exponential	4.3	10.3	38	51	83.5	57	0.0402	this paper
			Plateau	12.5	9.7	43	56	95.0	—	0.0464	this paper
			Regression	T_i 6	31.5	41	107	107	—	0.0657	this paper

DT: Doubling Time
LI: Labeling Index
GF: Growth Fraction
ϕ: Cell loss factor
R: Percentage of ^{3}HTdR reutilized by the cells
k_l: Hourly rate of cell loss

fected spleen is its rapid size decrease several days before death. Since the sudden increase of the number of circulating nucleated cells is well correlated with the decrease in spleen weight, it is suggested that splenic cells are suddenly released into peripheral blood by a mechanical or a biochemical process. Moreover, the calculation shows that this phenomenon is insufficient to explain the loss of splenic cells (approximately 9×10^8 cells per day for a spleen weighing 0.8 g) (see Mathematical Appendix 3), except if the circulating nucleated cells are endowed with a very short life-span. The erythroid maturation phenomenon is greatly enhanced at this stage of the disease—the percentage of reticulocytes reaching 26% (1)—but it can only partly explain the fate of the lost cells.

The high level of ^{3}HTdR reutilization measured in the rapid-growth phase (Table 3) indicates that rapid cell loss is associated with massive cell death. The reutilization of ^{3}HTdR can be demonstrated 10 hours after the labeling of the proliferating cells. This result suggests that cell death occurs early after labeling, i.e. it concerns primarily proliferating cells rather than mature cells. Furthermore, this result is compatible with death occurring during mitosis. Cytologic data (Table 1) is in agreement with preferential death of proliferating cells belonging to the erythroid cell line: indeed, in contrast to what is observed in the normal spleen, the proportion of young erythroid precursors is greater (30%) than that of more mature erythroblasts (20%) (acidophilic plus polychromatophilic blasts). Cytological data would also suggest that cell death involving myeloid precursors at all stages of maturation, as well as fibroblastic and reticular cells, cannot be excluded although it was not possible to measure with our techniques the cytokinetic parameters specific to these cell poupulations.

Thus, our results show that the MPSV-induced myeloproliferative syndrome shares many cytokinetic features with Friend and Rauscher leukemia: a) a rapid proliferation of most splenic blast cells, which does not decrease in the slow-growth phase of the spleen; b) a massive cell loss, cell death and ^{3}HTdR reutilization beginning at the exponential phase of spleen growth (Table 5).

It is probable that a certain proportion of cells leaving the spleen invade the liver. The number of neoplastic cells in this organ nevertheless remains very limited, especially at this phase of the disease (1). The invasion of the liver can thus explain the fate of only a very small proportion of the cells which leave the spleen.

As in Friend and Rauscher leukemia, it is likely that a certain percentage of cell loss is related to the production of erythroid and myeloid cells which leave the spleen and enter the peripheral blood. Since the number of nucleated cells in the peripheral blood increases very little in the rapid-growth phase, this type of cell loss probably involves mainly erythroid cells. Indeed, the percentage of reticulocytes is already equal to 5.0% compared to 2.5% in controls (1). Blood volume is not modified at this phase (Smadja-Joffe et al., unpublished results) and so it can be calculated, as described (3), that a maximum of 4×10^8 reticulocytes (Mathematical Appendix 2) are produced per day. Our cytokinetic data indicate that an average of 9×10^8 cells disap-

pear daily from an MPSV-infected spleen weighing 800 mg (Mathematical Appendix 3). Therefore, approximately 45% of cell loss would be due to in situ cell death. These values are rather comparable to those obtained in Friend leukemia spleen (respectively ⅓ and ⅔) (3).

The MPSV-induced neoplasia also exhibits cytokinetic differences compared to Friend and Rauscher leukemia. The GF during the rapid-growth phase of the MPSV-infected spleen is somewhat lower. This is probably due to the fact that erythroid cells, which are among the most intensively proliferating cells (14), are less numerous in the MPSV-infected spleen than in the Friend or Rauscher leukemic spleen. The cell loss factor is rather smaller. The drastic decrease observed in spleen weight a few days before death, is contrary to what occurs in Friend and Rauscher leukemia. To our knowledge, this is one of the first known examples in which a population of so-called neoplastic cells diminishes and this decrease is not associated with a cure of the disease but, inversely, with the rapid death of the infected mice.

To conclude, cellular proliferation in the MPSV-infected spleen is characterized by an intensive proliferation which is associated with massive cell death. These features are shared with those described in the case of Friend and Rauscher leukemic spleens.

MATHEMATICAL APPENDICES

1. *Calculation of daily DNA turnover in Friend leukemic spleen.* Splenic DNA turnover is described by an equation, $A_{24} = A_0 e^{\gamma 24}$ in which A_{24} is the splenic specific radioactivity 24 h after injection ^{3}HTdR, A_0 is the splenic specific activity at time 0, and T_{24} represents 24 h after injection of ^{3}HTdR. For $A_0 = 100$, $\gamma = 0.0564$, $A_{24} = 26$. This means that within one day, DNA associated radioactivity declines to 26% and that 74% of it leaves the spleen.

As demonstrated (19), the relationship $A_{24} = A_0 e^{k_l 24}$ is valuable if we hypothesize that cell loss occurs randomly in the proliferative as well as in the nonproliferative compartment. Using $k_l = 0.0402$, and $A_0 = 100$, we found that $A_{24} = 38$. This means that 38% of the specific radioactivity present at time 0 in the spleen remains in the organ after one day, or that 62% of it is lost.

2. *Daily production of reticulocytes in the peripheral blood at the exponential growth phase.* The calculation method has been published (3). For the present calculation, the following hematologic data were used (1):

a. hematocrit: 35%
b. percentage of reticulocytes: 5%
c. number of erythrocytes/mm^3: 6×10^6
d. life-span of reticulocytes: 40 h. This value corresponds to reticulocytes in mice infected with Anemiant-Friend virus. A value of 30 h, which is the life-span of normal reticulocytes, leads to final results which are not significantly different;
e. blood volume: to estimate a maximal value of reticulocyte production, a value of 8 ml/100 g body weight has been used.

3. *Daily cell loss in the rapid-growth phase.* The growth of the leukemic spleen is described by the equation: $\frac{dN(t)}{dt} = (k_p - k_l)N_t$ (1) in which N_t = cell number at time t.

It follows that the number N_{21} of cells produced per day is $N_{21} = \frac{k_1}{k_2 - k_1} N_0[(e^{(k_2-k_1)24} - 1)]$ (2), where N_0 = cell number at time 0. From equation (2), we see that $N_{21} = 1.04\ N_0$. The MPSV-infected spleen weighing 800 mg contains 8.8×10^8 cells (Table 1). Therefore $N_{21} = 1.04 \times 8.8 \times 10^8 = 9 \times 10^8$ cells.

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3. Cinétique de la prolifération cellulaire du sarcome induit par le virus de Moloney (M-MSV)

3.1. The cellular proliferation kinetics of the murine sarcoma induced by the Moloney Sarcoma Virus.

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F. Smadja-Joffe, M.C. Le Bousse-Kerdiles, B. Klein, E.P. Malaise, V. Degiorgis and C. Jasmin.

RESUME : Nous avons étudié la cinétique de la prolifération cellulaire dans le sarcome induit par le virus de Moloney dans les souris nouveaux-nés Swiss OFI. Par injection *in vivo* de thymidine tritiée et autoradiographie, nous avons montré que la majorité des cellules était en prolifération active (Index de marquage : 31% ; Coefficient de prolifération cellulaire : 78%). La durée moyenne du cycle mitotique était de 16 heures et la perte cellulaire était limitée, égale à 48%. L'étude de l'évolution en fonction du temps de l'activité spécifique de la tumeur après une injection unique de ^{3}HTdR ou de ^{125}IUdR, *in vivo*, n'a pas permis de démontrer l'existence d'une perte cellulaire telle qu'elle avait été calculée dans l'étude autoradiographique. Ce résultat peut être interprété par l'existence d'une réutilisation massive de la radioactivité en provenance des tissus sains.

CELLULAR PROLIFERATION KINETICS OF THE MURINE SARCOMA INDUCED BY MOLONEY SARCOMA VIRUS

F. SMADJA-JOFFE, M. C. LE BOUSSE-KERDILES, B. KLEIN
E. P. MALAISE*, C. JASMIN and V. DEGIORGIS.

ABSTRACT

Cell proliferation kinetics of the sarcoma induced by Moloney virus was studied in newborn Swiss OF1 mice.

After injection of tritiated thymidine *in vivo* followed by autoradiography, it was shown that the majority of cells were actively proliferating (Labelling Index was equal to 31%, Growth Fraction was equal to 78%). The mean cell cycle was 16 hours and cell loss was relatively low (Cell loss factor equal to 48%). The study of tumour specific activity with time after a single injection of ^{3}HTdR or ^{125}IUdR *in vivo* did not demonstrate cell loss as calculated by autoradiography. This result is consistent with a massive reutilization of radioactivity furnished by healthy tissues.

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INTRODUCTION

When injected in newborn mice, the Moloney Strain of Murine Sarcoma Virus (M-MSV) induces solid malignant tumours after several days which rapidly grow and kill the animals (Moloney, 1966). Histologically, these tumours are pleiomorphic sarcomas, in which mesenchymal, fibroblastic and granulocytic cells are primarily observed. Thus, these tumours have occasionally been classified as rhabdomyosarcomas (Moloney, 1966) and occasionally as atypical granulomas (Stanton *et al.*, 1968).

We have investigated the cellular proliferation kinetics of this sarcoma with two main purposes in mind. The study of cellular proliferation kinetics in several retrovirus-induced murine leukemias (Friend's leukemia, Rauscher's leukemia and MPSV-induced myeloproliferative syndrome) has shown the existence of massive cell loss and cell death, beginning with the initial phase of spleen growth (Smadja-Joffe *et al.*, 1973; Smadja-Joffe *et al.*, 1976; De Both, 1978; Smadja-Joffe *et al.*, 1981). We investigated whether another tumor, also induced by a retrovirus but which is solid, would also result in the same phenomenon. We also compared the main characteristics of cellular proliferation in neoplasias induced by M-MSV (present study) and by the MPSV (Smadja-Joffe *et al.*, 1980 a,b), since the M-MSV genome is still present in MPSV (Ostertag *et al.*, 1980). Siegler (1970) has suggested that the M-MSV-induced sarcoma was the site of massive cellular destruction provoked by the virus. Therefore, a quantitative study of cell loss and cell death was of special interest in order to evaluate the vali-

dity of this hypothesis.

MATERIALS AND METHODS

Swiss OF1 mice (Iffa-Credo, France) were used. They were specific pathogen-free and free of reovirus and Sendaï virus. The Moloney strain of Murine Sarcoma Virus (M-MSV) was used. The viral suspension was diluted 1/10 before intramuscular injection (0.1 ml per mouse) into the left posterior thigh of 3-day-old mice. Tumour and body weights of ten mice were determined daily starting on day 2 after virus injection and continuing until the first death occured. The tumours were dissected and then weighed.

The cytological study was performed on days 6, 8 and 11 after viral injection on 4 tumours per group. The percentages of tumour, granulocytic and lymphoid cells were determined on histological sections (3 µm thick, hematoxylin-eosin staining). The naphtol AS-D chloroacetate esterase specific reaction (Leder, 1970) was performed, in order to facilitate identification of the granulocytic cells.

The autoradiographic study was 6 days after infection: 0.5 µCi/g of ^{3}HTdR (CEA, France. Specific Activity 25 Ci/m mole) was injected intraperitoneally into MSV-infected mice. The percentages of labelled cells and of labelled mitoses were determined on histological sections using autoradiographic procedures previously described in detail

(Smadja-Joffe *et al.*, 1981). The labelling index (LI) and the mitotic index (MI) were calculated for 4000 tumoral cells, excluding polymorphonuclear (PMN) cells. The duration of the mitotic cycle (Tc) and of its distinct phases were determined from the PLM curve (representing the time-dependance of the Percentage of Labelled Mitosis) (Quastler and Shermann, 1959) and by the optimization procedure described by Valleron *et al.* (1973). The growth fraction (GF), cell loss factor (Ø) and the rates of cell loss and cell production (respectively k_l and k_p) were calculated as previously described (Smadja-Joffe *et al.*, 1981).

The reutilization of ^{3}HTdR and ^{125}IUdR was studied 6 days after injection. At time 0, 0.2 µCi/g of ^{125}IUdR (Radiochemical Center Amersham. 0.07 Ci/m mole) and 0.2 µCi/g ^{3}HTdR (CEA, France. 25 Ci/m mole) were intraperitoneally injected into 2 groups of 25 mice each. Drinking water contained 0.1% NaI. At different times (1, 42, 72 and 96 hours), five mice from each group were sacrificied and tumours were weighed after excision of the femur and skin. Specific radioactivities per g of tissue and per µg of DNA were determined as previously described (Smadja-Joffe *et al.*, 1981).

RESULTS

Tumour and body weight. A significant weight increase of the M-MSV infected thigh was observed 4 days after viral injection. By this time, the tumour was well visible in the thigh. The growth curve between days 4 and 9 can be fitted to an exponential line with a mean

doubling time (DT) of 37 hours. Tumour growth rate decreased after day 9 and the first deaths occured on day 13 (Fig. 1).

Cytological study. The population of tumour cells was composed of approximately equal proportions of two cell types: spindle cells with a central nucleus, and round cells often with an eccentric nucleus and a dense cytoplasm. Tumour cells represented approximately 60% of the cells present in the tumour and this figure did not vary significantly during the major part of tumour growth (Table 1). A minimum of 1000 cells per tumour was examined under oil immersion along three vertical and three horizontal axes (x 2500 magnification).

Cellular proliferation parameters. Both populations of tumour cells were labelled by ^{3}HTdR (Fig. 2B) and were characterized by equal LI values. From the PLM curve (Fig. 3), the duration of the mitotic cycle and of its various phases, as well as the major cytokinetic parameters, were determined (Table 2). The specific activity of the tumour, as cpm per gram of tissue or as cpm per microgram of DNA, did not decrease significantly after a single injection of ^{125}IUdR or ^{3}HTdR (Fig. 4).

DISCUSSION

The duration of the mitotic cycle determined during the exponential growth phase of the Moloney sarcoma is quite comparable to

the median observed by other workers for transplanted rat and mouse sarcoma (Table 3). Therefore, the viral etiology of the Moloney sarcoma does not appreciably affect the mitotic cycle of the cells.

The labelling index, and especially the growth fraction, reached values higher than the medians determined for transplanted sarcomas and for the few induced sarcomas which had been studied (Table 3). During the log-phase of the disease, however, the GF of the Moloney rhabdomyosarcoma was of the same order of magnitude as those measured in neoplastic spleens of Friend and Rauscher leukemias and was also similar to those determined in mice with the MPSV-induced myeloproliferative syndrome (Table 4). Nevertheless, these data do not appear to be sufficient to attribute these high GF values to the viral etiology of these various tumours.

The sarcoma induced by M-MSV did not lead to massive cell loss, contrary to neoplastic spleens of mice with Friend or Rauscher leukemia or to mice with the MPSV-induced myeloproliferative syndrome. In particular, the cellular proliferations induced by two very similar viruses, M-MSV and MPSV (Ostertag *et al.*, 1981; Smadja-Joffe *et al.*, 1981), are characterized by different levels of cell loss: Ø in the M-MSV-induced sarcoma is about half (Ø = 48%) that in MPSV-induced neoplastic spleens (Ø = 84%) (Table 4), indicating that the retrovirus does not directly cause massive cell loss.

The time-dependance of specific radioactivity in tumours following the injection of ^{3}HTdR of ^{125}IUdR (Feinendegen *et al.*, 1973) did not enable us to demonstrate a significant reduction of tumour specific radioactivity, regardless of the isotope. Tumour DT was 37 hours and the increase of the cell population alone should have diluted the isotope and reduced specific radioactivity to 25% after 74 hours. Autoradiography showed that cell loss reached 48% and so after 74 hours we should have detected a specific radioactivity much lower that 70% of the initial value, even providing that reutilization was 100%. An alternative explanation of our results is an influx of radioactivity from an extra-tumour source, *e.g.* the small intestine (external reutilization), this does not exclude the possibility of a partial or total reutilization from dying tumour cells (internal reutilization). A reutilization of IUdR has also been evoked by Begg to explain the increased specific activity observed in several tumours (Begg, 1977). Further experiments are in progress in our laboratory to elucidate this phenomenon.

The Moloney virus-induced sarcoma has been proposed as a model for human mesenchymal sarcomas (Perk and Moloney, 1966; Siegler, 1970). The present results show that the cellular proliferation kinetics of the Moloney sarcoma are very different from those of most human mesenchymal sarcomas studied (Table 3), which are generally characterized by low LI and GF values (Malaise *et al.*, 1973). The radiation or chemical induced sarcomas in rats and mice exhibit LI and GF values which are

much lower than those of the Moloney sarcoma and from a kinetic standpoint probably represent a more valid model for these human sarcomas.

In conclusion, cellular proliferation of the M-MSV-induced sarcoma is characterized by the intensive proliferation of tumour cells. This proliferation, unlike the neoplastic spleens of several retrovirus-induced murine leukemias, is not associated with massive cell loss.

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TABLE 1 : M-MSV INDUCED SARCOMA: EVOLUTION OF THE DIFFERENT CELL POPULATIONS

Days after viral injection	Tumor weight (mg)	% tumoral cells	% Granulocytic cells	% Lymphoid cells	% Indetermined cells
6	144 (19.8)	58.0 (6.7)	38 (8.8)	3.7 (1.2)	0.3 (0.03)
8	450 (130)	71.4 (5.2)	24.6 (6.3)	2.8 (0.8)	1.2 (0.02)
11	880 (60)	62.5 (5.2)	35.5 (4.8)	1.7 (0.9)	0.3 (0.03)

TABLE 2 : PARAMETERS OF CELLULAR PROLIFERATION OF THE M-MSV INDUCED SARCOMA

Days after virus injection	Mean tumor weight (mg)	DT (hr)	LI (%)	MI	T_{G1} (hr)	TS (hr)	T_{G2} (hr)	T_M (hr)	T_C (hr)	GF (%)	Ø (%)	k_p (hr^{-1})	k_1 (hr^{-1})
6	144 (20)	37	31.0 (2.5)	2.8 (0.7)	4.4 (2.1)	6.3 (3.6)	4.7 (3.5)	0.5 (0.0)	15.9 (9.2)	78	43.8	0.0362	0.0175

DT: doubling time of the tumor

LI: labeling index determined one hour after ^{3}HTdR injection

MI: mitotic index

T_{G1}, T_S, T_{G2}, T_M: duration of the presynthetic, DNA synthesis, post synthetic and mitotic phases

T_C: duration of the cell cycle

Ø: cell loss factor

GF: Growth Factor

k_p and k_1: hourly rates of cell production and cell loss

Standard deviation (SD) in parenthesis

TABLE 3 : CYTOKINETIC CHARACTERISTICS OF TRANSPLANTED AND INDUCED EXPERIMENTAL SARCOMAS AND OF HUMAN MESENCHYMAL SARCOMAS

Origin	Host	Designation	DT (hr)	LI (%)	GF (%)	T_C (hr)	Ø (%)	References
Frequently passaged Sarcomas	Rat	Fibrosarcomas (3) Osteosarcomas (1) Rhabdomyosarcomas (2) "Sarcomas" (4)	33*	26*	50*	16*	20*	Steel, 1977
	Mice	"Sarcomas" (4) Fibrosarcomas (1)						
Virus induced sarcomas	M-MSV Mice	Rhabdomyosarcomas	37	31	78	16	48	Smadja-Joffe, 1981
	Polyoma virus Rat	Sarcomas	47	13	39	21	16	Zobl, 1975
	DMBA Mice	Sarcomas	113	13	48	20	73	Denechaud, 1979
	^{144}Cerium Rat	Soft-tissue Osteosarcomas	168	10				
		Bone-tissue Osteosarcomas	432	6	[20]	18	[80]	Klein, 1977
		Fibrosarcomas	144	14				
Spontaneous	Human	Mesenchymal sarcomas	936	2 (0.3→6)	11		29	

Legends : - DT, LI, T_C, GF and Ø : see Table 2

- Values in brackets are less reliable
- Values in parenthesis indicate the number of studies which have been checked
- (*) These datas are the median values calculated from datas concerning murine and rat sarcomas

TABLE 4 : CYTOKINETIC CHARACTERISTICS OF VIRUS-INDUCED NEOPLASIA

Virus	Host	Classification	Proliferating cells and site of proliferation	DT (hr)	LI (%)	T_C (hr)	GF (%)	Ø (%)	k_{p_1} (hr^{-1})	k_{l_1} (hr^{-1})	references
Moloney Murine Sarcoma Virus (M-MSV) (retrovirus)	New-born Mice	Rhabdomyosarcoma	fibroblasts and mesenchymal cells (thigh)	37	31	16	78	48	0.036	0.0175	Smadja-Joffe *et al.* (present paper)
Murine Mammary Tumor Virus (MMTV) (retrovirus B particles)	Mice	Mammary Carcinoma	(breast)	108	16	16	46	71	0.0236	0.0171	Mendelsohn 1965
Polyoma virus (DNA virus)	Rat	Renal Carcinoma	(kidney)	47	13	21	39	16	0.0156	0.0008	Zobl *et al.* 1975
Polycythemia inducing Friend Virus (VFP) (retrovirus)	Mice	erythroblastosis	erythroblasts (spleen)	144	45	8	64	94	0.0618	0.0686	Smadja-Joffe *et al.* 1973 - 1976
Rauscher Virus (RLV) (retrovirus)	Mice	erythroblastosis	erythroblasts (spleen)	156	51	8	85	96	0.0768	0.046	De Both 1978
Myeloproliferative Sarcoma Virus (MPSV) (retrovirus)	Mice	Myeloproliferative syndrome	erythroblasts + myeloblastic cells + fibroblasts (spleen)	103	38	10	51	84	0.0412	0.0402	Smadja-Joffe *et al.* 1980 a, b

Legends : see Table 2

FIGURES LEGENDS

Figure 1: Tumour growth curve. Time 0 equals injection of M-MSV. Each point represents the mean ± S.D. calculated from ten mice.

Figure 2: 2A : Histological section of a tumour weighing 700 mg. Hematoxylin and eosin staining x 500

➤: spindle tumor cell

⇨: "round"-shaped tumour cell

⟶: granulocytic cell

2B : Autoradiogram of a tumor labeled with ^{3}HTdR (0.5 µCi/g ; 25 Ci/mMole)

➤: labeled spindle tumour cell

⇨: labeled "round-shaped" tumour cell

Hematoxylin and eosin staining x 1250

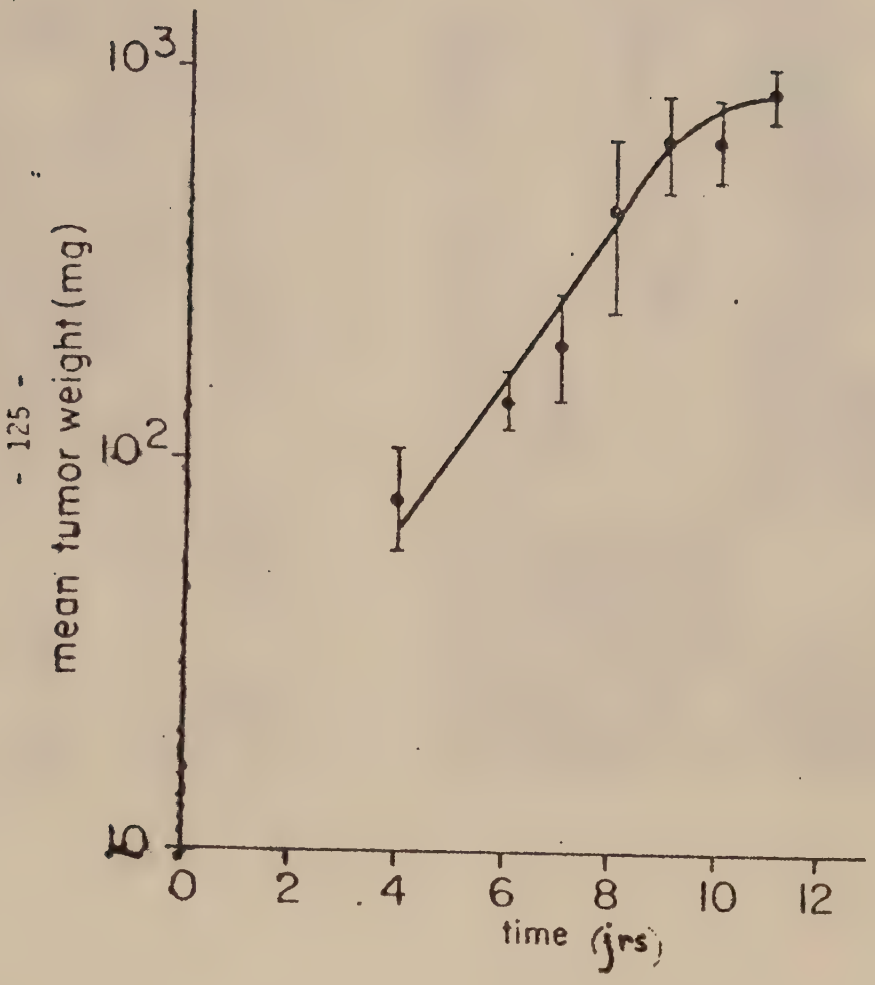
Figure 3: Percentage of labeled mitoses (PLM) curve of M-MSV induced tumor during the exponential growth phase. Tritiated thymidine (0.5 µCi/g) was injected at time 0. At least 200 mitoses at each point were counted. The curve was optimized with a mathe-model (see text).

Figure 4: Specific activity in M-MSV induced sarcoma after a single injection of 0.2 µCi/g ^{3}HTdR (o----o) or 0.2 µCi/g ^{125}IUdR (●——●). The points represent mean, expressed as % of those measured one hour after isotope injection. Means were calculated from data of four independent experiments. Five tumors per group were used. The vertical bars represent 2 standard deviations of

the mean (95% confidence limits). Data were plotted on semi-logarithmic coordinates and exponential regression curves were generated.

A) Specific activity as cpm/g of tumor

B) Specific activity measured as cpm/µg of DNA.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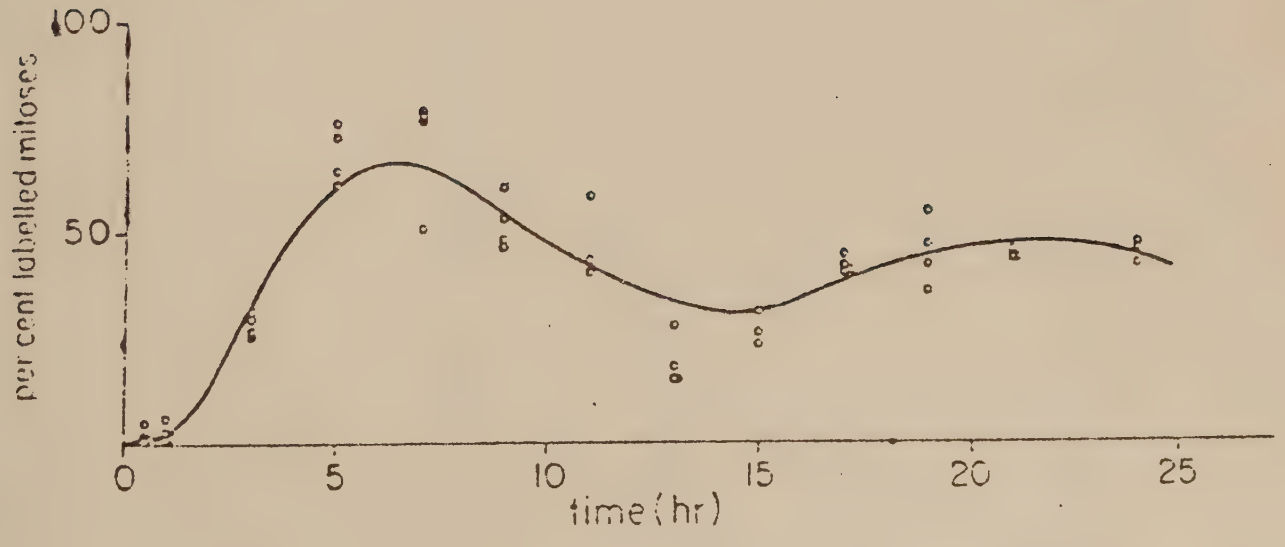
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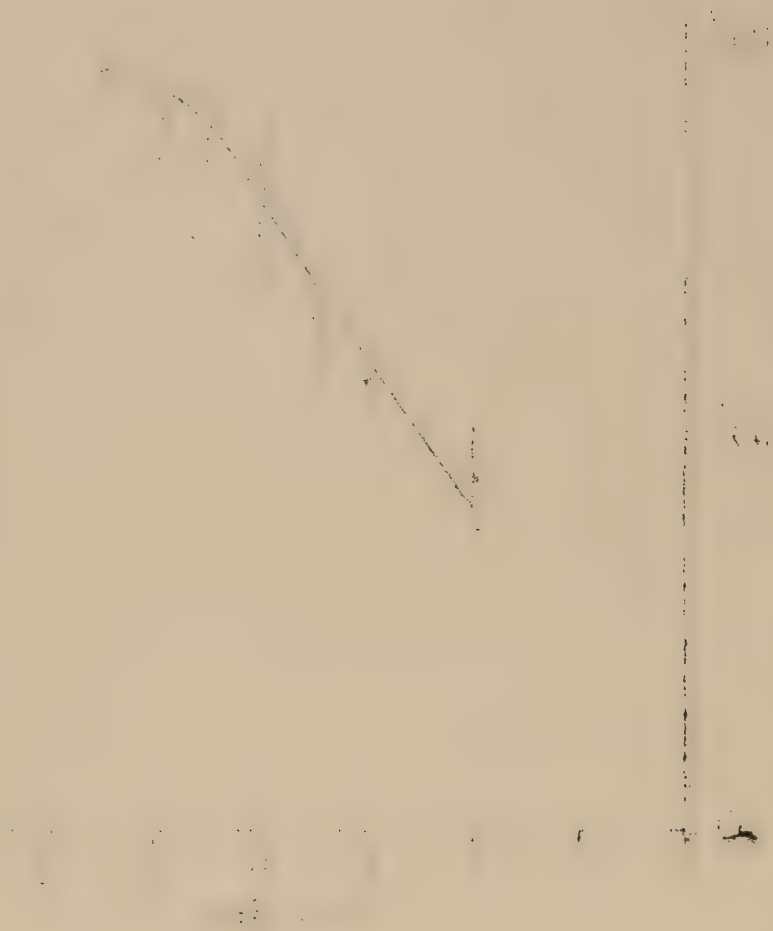
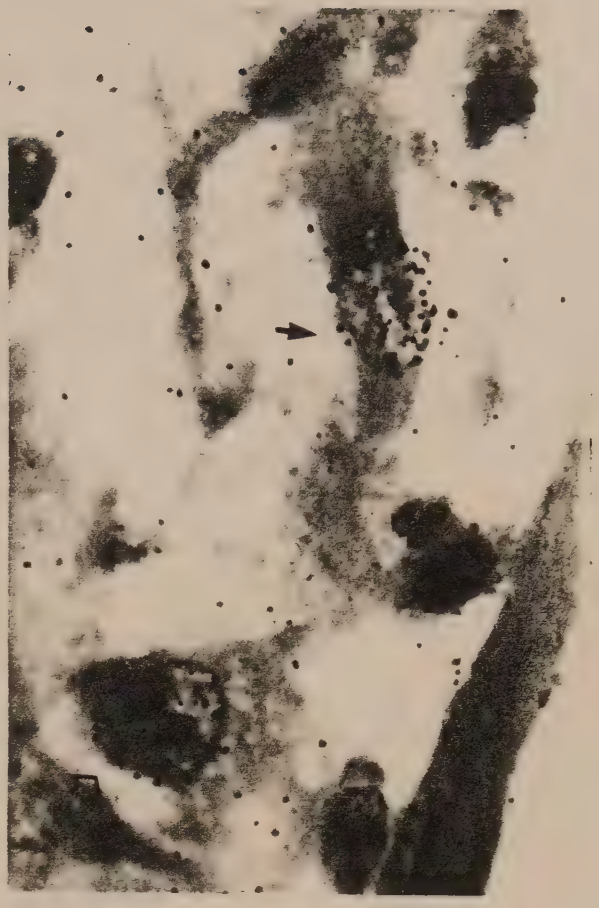
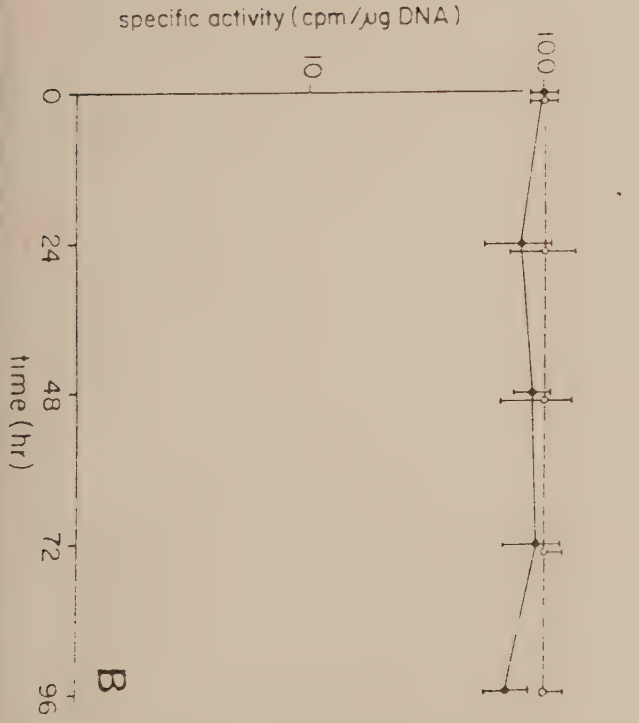
time (hr)



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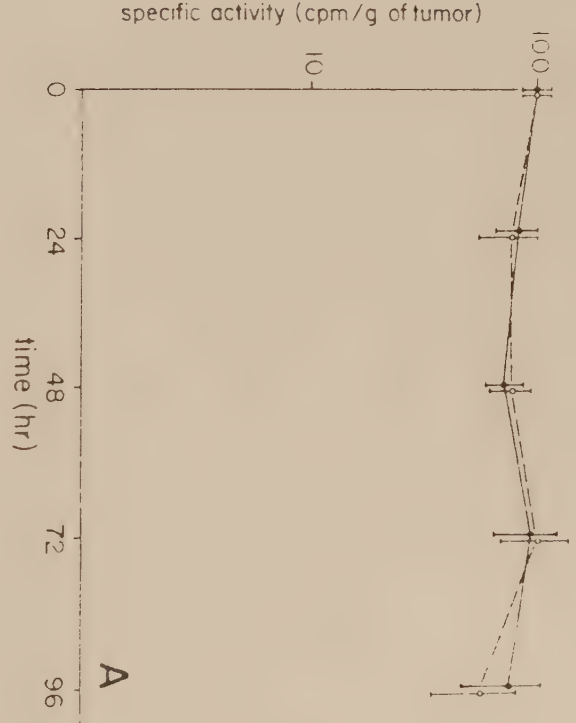
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DISCUSSION

Nous avons étudié la cinétique de la prolifération cellulaire de trois néoplasies murines induites par des rétrovirus : deux hémopathies malignes, la leucémie de Friend et le syndrôme myéloprolifératif induit par le MPSV, et une tumeur solide, le sarcome induit par le M-MSV.

Nos résultats ont montré que les deux hémopathies présentaient des caractéristiques communes : la prolifération des cellules spléniques y est caractérisée par un index de marquage et un coefficient de prolifération cellulaire élevés, pendant la phase de croissance rapide de la rate néoplasique et même lorsque le poids de celle-ci n'augmente plus que très lentement. D'autre part, une perte cellulaire particulièrement élevée (environ 90%) est observée dès la phase de croissance rapide de la rate, et elle est dûe pour une grande part (50% à 75% environ) à la mort massive, *in situ*, des cellules proliférantes(93, 94, 122).

Notre étude de la cinétique de la prolifération cellulaire du sarcome induit par le M-MSV montre cependant qu'une perte cellulaire de cette importance n'est pas une caractéristique commune à l'ensemble des néoplasies induites par des rétrovirus, puisque, dans cette tumeur solide, elle ne dépasse pas 45% (123).

Nous nous proposons à présent de discuter, d'une part dans le cas des leucémies, d'autre part dans le cas des tumeurs solides, de l'influence éventuelle de l'étiologie et/ou de la physiopathologie de ces 3 néoplasies sur

la cinétique de leur prolifération cellulaire. Nous essaierons également de montrer comment les résultats que nous avons obtenus contribuent à une meilleure compréhension de la physiopathologie des hémopathies malignes étudiées.

1. INFLUENCE DE L'ETIOLOGIE ET DE LA PHYSIOPATHOLOGIE SUR LA CINETIQUE DE LA PROLIFERATION CELLULAIRE

1.1. Hémopathies malignes

La cinétique de la prolifération cellulaire a surtout été étudiée dans les leucémies greffées, et en particulier dans la leucémie LI210 (26, 27, 30, 31, 32). Or, si les cellules LI210 conservent encore certaines des caractéristiques propres aux cellules hématopoiétiques, la maladie provoquée par leur injection à des souris ne présente pas les caractéristiques des leucémies spontanées. En effet, ces cellules ne se multiplient pas préférentiellement dans les organes hématopoiétiques, et on n'observe pas de perturbation progressive de l'hématopoièse normale. Au contraire, les cellules LI210 se multiplient *in situ*, provoquant une ascite ou une tumeur selon qu'elles ont été injectées par voie intrapéritonéale ou sous-cutanée. Un tel modèle s'apparente donc davantage à une culture *in vivo* de cellules transformées qu'à une leucémie. La différence fondamentale qui existe entre ces deux types de néoplasies improprement désignées par le même vocable, est souligné par le très faible taux de pertes cellulaires pendant la phase de croissance rapide de la leucémie LI210 (26, 30, 32), alors que ces pertes avoisinent 90% dans les hémopathies malignes viro-induites que nous avons étudiées (93, 94, 122).

Un éventuel rôle spécifique du virus sur la prolifération cellulaire n'a pû être mis en évidence, car la cinétique de la prolifération cellulaire n'a été étudiée dans aucune leucémie expérimentale induite par d'autres agents oncogènes (radiations, substances chimiques...) (124, 125, 126). Les seules études concernant la cinétique de la prolifération cellulaire ont été effectuées dans deux hémopathies malignes :

- le lymphome spontané des souris AKR, dû au virus de Gross (127) ; cette étude très limitée, indique que la population des grands lymphoblastes proliférant dans le thymus est caractérisée par un index de marquage élevé et une durée de cycle cellulaire courte (Tableau 7).

- la leucémie érythroblastique induite par le virus de Rauscher (95). Comme dans la leucémie de Friend, la prolifération cellulaire dans la rate est intensive et associée à une perte cellulaire massive, résultant d'une mort rapide *in situ* de cellules proliférantes et d'une différenciation d'une partie d'entre elles en érythrocytes de courte durée de vie.

Nous nous contenterons donc de remarquer que les trois hémopathies qui ont été étudiées : la leucémie de Friend (93, 94), la leucémie de Rauscher (95) et le syndrôme myéloprolifératif induit par le MPSV (122), présentent en commun non seulement d'importantes caractéristiques de la cinétique de la prolifération cellulaire, mais également de nombreuses caractéristiques physiopathologiques. En effet, ce sont des néoplasies qui

apparaissent sans phase de latence ou avec une latence très brève après l'infection virale. Elles évoluent rapidement, provoquant généralement la mort des souris en moins de 60 jours environ (7, 8, 10, 19). Le nombre de cellules souches hématopoiétiques est très augmenté (76, 77, 78, 84, 84, 86, 91, 128, 129). Les cellules leucémiques conservent la capacité de se différencier (20, 22, 91, 92, 95) et sont difficilement greffables (7, 23, 24, 25, 87, 88). Il est donc possible que, dans certaines néoplasies, une corrélation puisse être établie entre des caractéristiques de la cinétique de la prolifération cellulaire et celles de la physiopathologie.

1.2. Sarcomes

Nous avons étudié la cinétique de la prolifération cellulaire du sarcome induit par le M-MSV principalement dans le but de déterminer si, dans cette tumeur solide, nous observerions, du fait de son étiologie virale, les mêmes caractéristiques de prolifération cellulaire que dans les hémopathies malignes viro-induites précédemment étudiées.

Nos résultats indiquent en effet que, comme dans celles-ci, et contrairement aux sarcomes induits et aux sarcomes humains étudiés jusqu'à présent, la prolifération cellulaire y est très intensive (Index de marquage et coefficient de prolifération cellulaire élevés) (Tableau 8) (123, 130, 131, 132, 133). Mais, trop peu de sarcomes viro-induits ont été étudiés pour qu'il soit possible d'établir une corrélation entre cette caractéristique cinétique et l'étiologie virale de cette tumeur. D'autre part,

Tableau 7 : CARACTERISTIQUES DE LA CINETIQUE DE LA PROLIFERATION CELLULAIRE DES NEOPLASIES INDUITES PAR DES VIRUS CHEZ LA SOURIS OU LE RAT.

	Virus	Hote	Classification	Cytologie et (localisation)	DT (heures)	LI (%)	TC (hr)	GF %	Ø %	kp h^{-1}	k_1 h^{-1}	Reference
Hémopathies	Gross	Souris	Lymphome	- lymphoblastes - (thymus)		30à 86	8					127
	Friend VFP	Souris	Erythroblastose	- cellules érythroïdes (rate)	144	45	8	64	94	0.062	0.069	93-94
	Rauscher RLV	Souris	Erythroblastose	- cellules érythroïdes (rate)	156	51	8	85	96	0.077	0.046	95
	MPSV	Souris	Syndrome myéloprolifératif	- cellules érythroïdes et granulocytaires (rate)	103	38	10	51	84	0.041	0.040	122
Tumeurs solides du tissu conjonctif ou épithélial	M-MSV Moloney Murine Sarcoma Virus	Souris nouveau-né	Rhabdomyosarcome	- fibroblastes et cellules mésenchymales (muscle)	37	31	16	78	48	0.036	0.0175	123
	MMTV Murine Mammary Tumors Virus	Souris	Carcinome mammaire	- cellules glandulaires (glande mammaire)	108	16	16	46	71	0.0236	0.0171	101
	Polyoma Virus	Rat	Sarcome	- cellules conjonctives (rein)	47	13	21	39	16	0.0156	0.0008	130

Tableau 8 : CINETIQUE DE LA PROLIFERATION CELLULAIRE DE SARCOMES INDUITS ET TRANSPLANTES CHEZ LE RAT ET LA SOURIS ET DES SARCOMES HUMAINS.

Origine		Hôte	Nombre et classification	DT(h)	LI %	GF %	Tc(h)	Ø %	Reference
tumeurs ayant été transplantées de multiples fois		Rat (10)	Fibrosarcomes (3), ostéosarcomes (1), rhabdomyosarcomes (2), "sarcomes" (4)	33 *	26 *	50 *	16 *	20 *	96
		souris	"sarcomes" (4), fibrosarcomes (1)						
sarcomes induits	virus M-MSV	souris (nouveau-né)	rhabdomyosarcome	37	31	78	16	48	123
	virus polyoma virus	rat	sarcome	47	13	39	21	16	130
	DMBA	souris	sarcome	113	13	48	20	73	131
	^{144}Cérium	rat	ostéosarcomes des tissus mous	168	10				
			ostéosarcomes des parties dures	432	6	[20]	18	[80]	132
			fibrosarcomes	144	14				
	Spontanée	homme	sarcomes mésenchymateux	936	2 (0.3 → 6)	11		29	

DI : Temps de doublement
LI : Index de marquage
GF : Coefficient de proliferation cellulaire
Tc : durée du cycle mitotique
Ø : taux de perte cellulaire

- Les valeurs entre crochets [ ] sont moins fiables.
- Les valeurs entre parenthèses indiquent le nombre d'études effectuées.
* Ces chiffres sont les médianes calculées à partir des 15 valeurs individuelles

et contrairement à ce qu'avaient suggéré les études histologiques, la perte cellulaire reste très limitée dans ce sarcome, du même ordre de grandeur que la médiane calculée à partir des valeurs mesurées sur 15 sarcomes multitransplantés (96). Nous n'avons pû quantifier la mort cellulaire dans le sarcome induit par le M-MSV, car nous avons observé une importante réutilisation de l'^{125}IUdR (122). Une réutilisation de l'IUdR a également été décrite récemment dans quelques tumeurs solides (134, 135). Nos résultats confirment que malgré l'intérêt que présente cette molécule pour l'étude de la mort cellulaire, son utilisation ne peut être généralisée à l'étude de toutes les tumeurs. Une étude est en cours actuellement , afin de déterminer si la réutilisation massive que nous avons observée, résultait d'un afflux d'^{125}IUdR en provenance des tissus sains en prolifération active (intestin par exemple), ou si l'^{125}IUdR était libéré par les cellules tumorales elles-mêmes.

2. CONTRIBUTION DES ETUDES DE LA CINETIQUE DE LA PROLIFERATION CELLULAIRE A LA COMPREHENSION DE LA PHYSIOPATHOLOGIE DES HEMOPATHIES MALIGNES EXPERIMENTALES

La complexité des mécanismes aboutissant à la transformation maligne des cellules du système hématopoiétique est telle que son étude nécessite des approches expérimentales variées : biochimiques, physiologiques etc... L'une d'elles est l'étude de la cinétique de la prolifération cellulaire.

Nous montrerons que dans le cas de la leucémie de Friend, cette étude a été particulièrement fructueuse puisqu'elle a permis de préciser un modèle rendant compte de façon cohérente des principales données expérimentales.

2.1. La leucémie de Friend

L'étude de la cinétique de la prolifération des cellules leucémiques de Friend montre que seulement le tiers d'entre elles se différencie le long de la lignée érythroïde et qu'environ les deux-tiers meurent rapidement dans la rate, après seulement une ou deux divisions. D'autre part, ces cellules ne présentent pas les caractéristiques qui permettent actuellement de définir une cellule maligne (136) : elles n'ont ni la capacité de se greffer facilement *in vivo*, ni anomalie chromosomique détectable (23, 24, 25, 87, 88, 137).

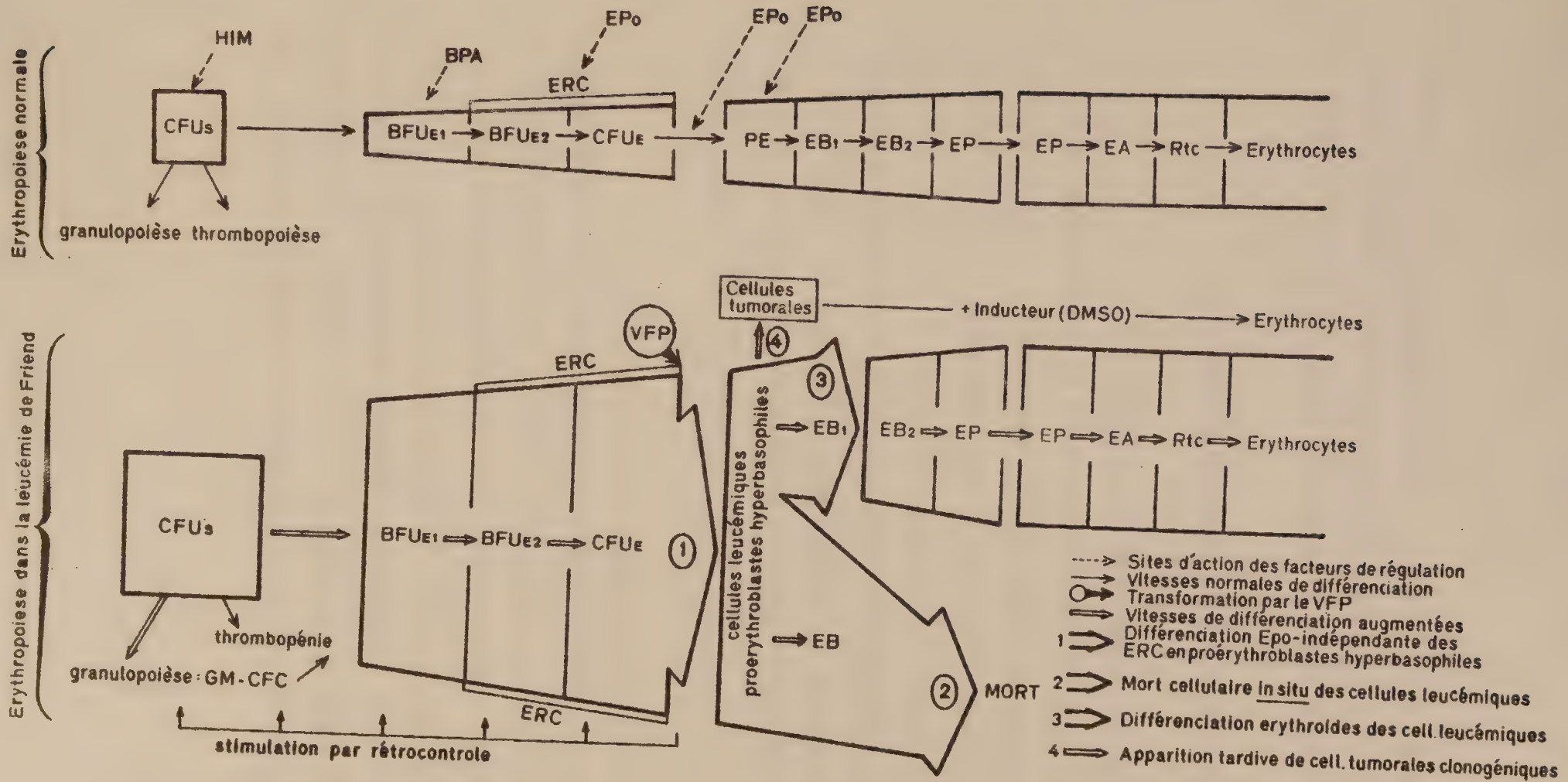
Elles sont donc très différentes des cellules tumorales de Friend, qui ont servi de matériel pour l'étude de la régulation moléculaire de la synthèse de l'hémoglobine (138, 139). En effet, ces cellules n'ont pû être sélectionnées qu'à une phase tardive de la maladie (23, 24, 25). Elles sont facilement greffables, donnent naissance à des lignées cellulaires *in vitro* (137, 138) et présentent des anomalies chromosomiques (139). Leur différenciation érythroïde est bloquée à différents stades (140, 141, 142). Les relations existant entre l'infection virale et l'apparition de ces cellules tumorales ne sont pas bien connues actuellement (142, 143, 144).

L'ensemble des propriétés des cellules leucémiques de Friend suggère donc que ces cellules, bien que proliférantes, n'ont pas la capacité de s'autorenouveler. Comment donc expliquer l'accroissement constant de la taille de cette population au cours de la maladie ?

Dans les conditions normales, la différenciation des ERC en proérythroblastes ne peut s'effectuer qu'en présence d'érythropoiétine, et c'est le taux de cette hormone qui en assure la régulation (16). Chez les souris présentant la leucémie de Friend, au contraire, la différenciation des ERC transformées peut s'effectuer sans érythropoiétine (84, 85, 86, 21, 22). Cette étape de l'érythropoièse n'est donc plus régulée. L'érythropoièse étant un processus en équilibre instable, la différenciation permanente des ERC transformées stimulerait leur production à partir des compartiments des cellules souches précurseurs (59, 67). Nous avons montré qu'environ 94% des cellules hyperbasophiles ainsi produites meurent rapidement (soit *in situ* après une ou deux divisions, soit après s'être différenciées en érythrocytes (93, 94). La splénomégalie due à l'augmentation progressive de leur nombre au cours de la maladie résulterait donc d'un excès de la vitesse de leur production par rapport à la vitesse de leur perte. Ce modèle de la leucémie induite par le virus de Friend (VFP) est représentée dans la Figure 9.

Des résultats récents tendent à confirmer ce modèle. En effet, grace à des techniques permettant de détecter un nombre très faible de cellules clonogéniques (10 contre 10^5 environ par la technique

Figure 9 : MODELE DE LA LEUCEMIE DE FRIEND



Abbreviations : Voir tableau 2

PE : Proérythroblastes

EB : Erythroblastes basophiles

EP : Erythroblastes polychromatophiles

EA : Erythroblastes acidophiles

Rtc : Réticulocytes

classique de la greffe sous-cutanée), il n'a été possible de déceler les premières cellules tumorales que 10 à 15 jours après l'injection du VFP, le poids de la rate ayant déjà été multiplié par 10 environ (147).

2.2. Le syndrôme myéloprolifératif induit par le MPSV

Nous avons montré que la prolifération des cellules splèniques dans la rate de souris présentant la néoplasie induite par le MPSV, a de nombreuses caractéristiques communes avec celle des cellules leucémiques de Friend : la perte cellulaire y est massive pendant la phase de croissance rapide de la rate ; cette perte est à la fois dûe à une mort cellulaire rapide *in situ*, et à une maturation hématopoiétique des cellules néoplasiques. D'autre part, cette néoplasie a de nombreuses caractéristiques physiologiques identiques à celles de la leucémie de Friend (Tableaux 3 et 4) (10, 91, 122).

On peut donc proposer, comme base de réflexion, un modèle ressemblant à celui proposé pour la leucémie de Friend. Dans ce modèle, l'augmentation du nombre de cellules néoplasiques dans la rate serait dûe à leur production continue, sous l'action du MPSV, à partir de cellules souches précurseurs. Cette production massive de cellules leucémiques ne pourrait être totalement compensée par leur mort et leur maturation hématopoiétique. Cependant, il est à prévoir que le modèle rendant compte de l'ensemble des résultats concernant ce syndrôme myéloprolifératif sera

plus complexe que celui de la leucémie de Friend. En effet, on y observe non seulement une érythroblastose mais également une transformation des cellules de la lignée granulocytaire, l'apparition de nodules tumoraux, et peut être une modification du microenvironnement splénique (10, 91, 92).

3. CONCLUSIONS

Nos études ont permis de compléter l'étude de la physiopathologie de leucémies pour lesquelles la connaissance du coefficient de la prolifération cellulaire, de la vitesse de prolifération et de la durée de vie des cellules leucémiques était importante. Le meilleur exemple en est la leucémie de Friend, où ces études, associées à la connaissance que nous avions des perturbations de l'érythropoièse dans cette maladie, nous ont permis de proposer un modèle cohérent de cette néoplasie.

D'autre part, ces études nous ont fait observer un phénomène qui intrigue beaucoup les cancérologues et les cinéticiens : il s'agit de la mort cellulaire. Pour mettre en évidence ce phénomène, nos études étaient indispensables et nos résultats soulignent combien l'histologie classique est une technique inappropriée à l'étude de ce phénomène. En effet, nous avons démontré l'existence d'une mort cellulaire massive dans une tumeur où elle n'était pas soupçonnée (rate leucémique de Friend), alors que dans le sarcome de Moloney, où l'histologie suggérait une lyse

cellulaire importante, notre étude démontre qu'elle reste limitée.

Cependant, si la connaissance de la cinétique de la prolifération cellulaire apparaît indispensable à une compréhension de la physiopathologie des néoplasies expérimentales, son étude est actuellement très handicapée par les problèmes techniques qu'elle pose. L'autoradiographie est en effet une technique longue : plusieurs semaines sont nécessaires à l'obtention d'autoradiogramme, leur interprétation exige l'observation d'un grand nombre de cellules. C'est pourquoi, plusieurs années de travail nous ont été nécessaires pour aboutir à des résultats fiables et nous n'avons étudié que 3 néoplasies viro-induites alors que, idéalement, nous aurions souhaité étendre notre étude à d'autres néoplasies, induites par des virus et par d'autres agents carcinogènes. Nous aurions ainsi pû déterminer si certaines des caractéristiques observées étaient spécifiquement provoquées par les rétrovirus, ou si elles étaient liées à une certaine physiopathologie.

Notre contribution aura donc été d'ajouter, à la liste des néoplasies expérimentales dont la prolifération cellulaire a été étudiée, les données concernant celle de trois néoplasies viro-induites, et d'avoir démontré l'intérêt de telles études pour une compréhension de leur physiopathologie.

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